

**The effect of welding fumes and
smoking on host-pathogen
interactions in bacterial
pneumonia**

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**A thesis submitted in partial fulfilment of the
requirements of the degree of Doctor of
Philosophy**

Queen Mary University of London

Statement of Originality

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Abstract

Background: Epidemiological evidence supports a strong association between exposure to inhaled toxins and adverse respiratory outcomes such as pneumococcal pneumonia and chronic respiratory conditions such as chronic obstructive pulmonary disorder (COPD). Chronic exposure to airborne particulate matter in occupational settings, such as welding, has been shown to reversibly increase risk of pneumococcal pneumonia. However, biological plausibility of this has not been shown and the molecular mechanisms are unknown. Chronic cigarette smoking causes reduced lung function and increased morbidity in COPD patients. These patients are highly vulnerable to viral and secondary pneumococcal infections. The molecular mechanisms are unclear.

Methods: Association between exposure to welding fumes (WF) and susceptibility to pneumococcal infection, rhinoviral infection and rhinoviral+secondary pneumococcal infection was assessed in lower airway and nasal epithelial cells *in vitro*. The role of the platelet activating factor receptor (PAFR), which is an entry receptor for the pneumococcus, was also assessed. Using two methods of WF exposure, susceptibility to pneumococcal infection following acute and chronic exposure was assessed in mice. Finally, PAFR expression was examined in the lungs of non-smokers, welders, smokers and smokers with COPD.

Results: Exposure of alveolar, bronchial and nasal epithelial cells to WF significantly increases pneumococcal invasion of these cells in a PAFR dependent manner. Exposure to WF increases susceptibility to infection by rhinovirus and vulnerability to secondary pneumococcal infections in a PAFR dependent manner. Exposure to WF increases susceptibility of mice to pneumococcal infection and PAFR mRNA expression levels in the lungs. Finally, PAFR mRNA levels are elevated in smokers with COPD compared with non-smokers. PAFR protein expression in the lungs of smokers with COPD is localised to the bronchial epithelium and bronchial glands.

Conclusions: PAFR is a potential anti-infective agent in pneumococcal pneumonia caused by exposure to WF and in COPD caused by chronic exposure to smoking.

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Publications

Peer reviewed publications:

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Suri R, Jose R, Wilson R, Brown J, Palmer K, Coggon D, Grigg J. **Exposure to mild steel welding fume and susceptibility to pneumococcal infection in mice**. European Respiratory Society 2013.

Suri R, Greiller C, Martineau A.R, Grigg J. **Treatment of alveolar epithelial cell line A549 with Vitamin D and Rhinovirus infection**. The Macrae Foundation International Symposium on Respiratory Viral Infections 2013.

Suri R, Palmer K, Ross J, Coggon D, Grigg J. **Exposure to welding fume and adhesion of Streptococcus pneumoniae to A549 alveolar cells**. British Thoracic Society Winter meeting 2012.

Suri R, Mushtaq N, Waite R, Grigg J. **Additive effect of air pollution particulate matter and cigarette smoke on pneumococcal adhesion to lower airway cells**. European Respiratory Society 2012.

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List of abbreviations

A549	alveolar epithelial cell line
BALF	broncho-alveolar lavage fluid
Bcl2	B-cell lymphoma 2
BEAS-2B	bronchial epithelial cell line
BHI	brain heart infusion
C/EBP δ	CCAAT enhancer binding protein δ
CD	cluster of differentiation
CFU	colony forming units
ChoP	phosphorylcholine
COPD	chronic obstructive pulmonary disease
CSC	cigarette smoke condensate
CSE	cigarette smoke extract
CV-3988	PAFR blocker
DAB	3, 3' diamino benzidine
DAPI	4',6-diamidino-2-phenylindole, dilactate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DRP	Disease Reduction Programme
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FEV1	forced expiratory volume in 1 second
FVC	forced vital capacity
G	L-glutamine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

GMA	gas metal arc
GM-CSF	granulocyte macrophage-colony stimulating factor
h	hours
H. influenzae	Haemophilus influenzae
hBD	human β defensin
IC	iota carrageenan
ICAM-1	intracellular adhesion molecule 1
IFN	interferon
IgG	immunoglobulin
IL	interleukin
L. monocytogenes	Listeria monocytogenes
LDH	lactate dehydrogenase
LDL	low density lipoprotein
LGG	lactobacillus rhamnosus GG
LPS	lipopolysaccharide
M. pneumoniae	Mycoplasma pneumoniae
MAP	mitogen activated protein
MDA5	melanoma differentiation-associated protein 5
MERTK	MER tyrosine kinase
MMA	manual metal arc
MMP	matrix metalloproteinase
MOI	multiplicity of infection
mRNA	messenger RNA
MS	mild steel
NAC	N-acetyl cysteine
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
Nod2	nucleotide-binding oligomerization domain-containing protein 2

Nrf2	nuclear factor erythroid-2-related factor 2
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
<i>P. gingivalis</i>	<i>Porphyromonas gingivalis</i>
P/S	penicillin-streptomycin
PAF	platelet activating factor
PAFR	platelet activating factor receptor
PBS	phosphate buffered saline
PM	particulate matter
poly I:C	polyinosinic:polycytidylic acid
PspA	lipoprotein pneumococcal surface adhesion
RIG-1	retinoic acid-inducible gene 1
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute-1640 media containing HEPES
RSV	respiratory syntial virus
RT-PCR	reverse transcription polymerase chain reaction
RV	rhinovirus
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. pneumoniae</i>	<i>Streptococcus pneumoniae</i>
S-CMC	S-carboxymethylcysteine
SM	squamous metaplasia
SS	stainless steel
TGF- β 1	transforming growth factor β 1
TNF- α	tumour necrosis factor α
VEGF	vascular endothelial growth factor
WF	welding fume
WHO	World Health Organisation

Chapter 1: Introduction

1.1 Environmental and occupational exposure to airborne particulate matter and respiratory infections

Pneumonia was first described as a disease by Hippocrates in the 4th century BC. However, it wasn't until the 19th century that Edward Klebs observed bacteria in the airways of people who had died from pneumonia and Carl Friedlander and Albert Frankel identified *Streptococcus pneumoniae* (*S. pneumoniae*) as a bacterial cause of the disease.

Pneumonia is the cause of death in 1 in 5 children in the developing world and is a major cause of morbidity in the developed world. *S. pneumoniae* is the most common causative agent of pneumonia¹⁻³. Although it causes invasive disease, the Gram positive bacterium is carried by a large proportion of the population without any symptoms of disease.

Viral pneumonia is caused by viruses such as respiratory syncytial virus (RSV) and rhinoviruses (RVs). Viral pneumonia is difficult to diagnose and is not commonly associated with fatalities. However, viral infections may predispose susceptible individuals to secondary bacterial infections which may further exacerbate the pneumonia, causing increased risk of death.

Welding, which has previously been a target occupation for the Disease Reduction Programme in the UK, has been associated with pneumonia, occupational asthma and cancer^{4,5}. Currently, several million people worldwide are full time occupational welders.

In this thesis the focus is on the effect of inhaled toxin exposure on interactions between the host and *S. pneumoniae* and human rhinoviruses, and on rhinoviral/pneumococcal interactions during co-infection of the host, in the context of bacterial pneumonia. In this chapter, the literature around *S. pneumoniae* and rhinovirus infection and viral-bacterial co-infections will first be reviewed. Then, the

evidence of interactions between sources of air pollution and infection will be reviewed.

1.1.1 *Streptococcus pneumoniae*: importance of adherence

Adherence of pneumococci to airway epithelial cells is the first step of pathogenesis^{6,7}. Proteins found on the surface of the pneumococcus called adhesins mediate this adherence. One such adhesin is phosphorylcholine (ChoP) which has strong structural similarities to the human platelet activating factor (PAF) and is recognised by and binds the PAF receptor (PAFR). PAFR is a G coupled receptor which is rapidly internalised after it binds its ligand⁸. This endocytosis has been shown to be mediated by β arrestin 1 which targets PAFR to clathrin coated pits⁹. Hence, *S. pneumoniae* co-opts PAFR for entry into human cells. It has been shown that in respiratory epithelial cells infection with *S. pneumoniae* induces expression of the transcription factor, CCAAT enhancer binding protein δ (C/EBP δ), which in turn induces the expression of PAFR, as shown in figure 1¹⁰. PAFR mRNA and protein expression was observed in nasal epithelial cells purified from the nasal mucosa of human subjects. Expression was also observed in submucosal glands¹¹. Other adhesins present on the pneumococcal surface, such as lipoprotein pneumococcal surface adhesin (PspA), have also been shown to be involved in mediating the attachment of bacteria to both nasopharyngeal and lower airway epithelial cells¹².

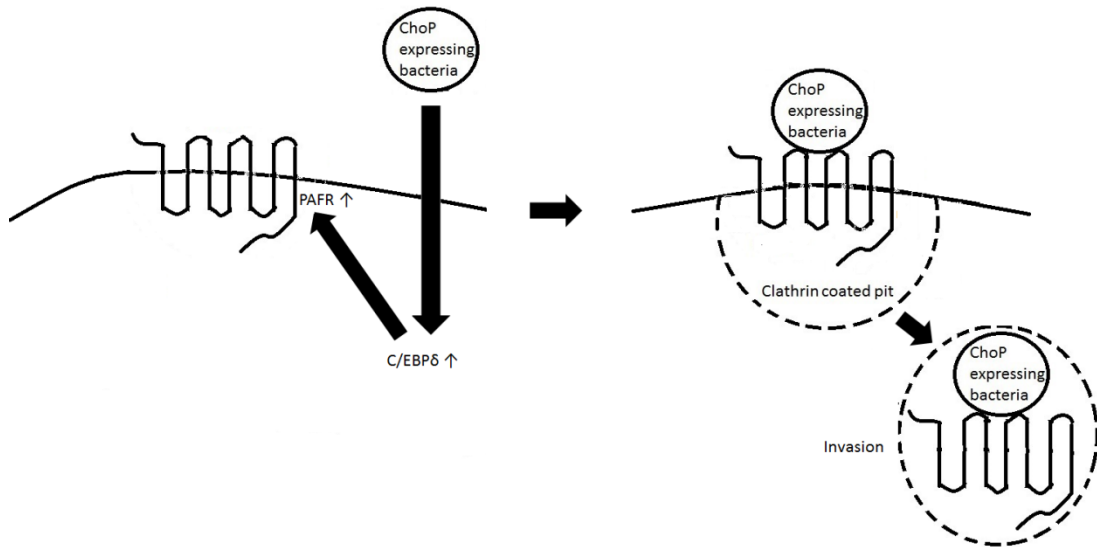


Figure 1: *S. pneumoniae* co-opts PAFR for entry into human epithelial cells. *S. pneumoniae* induces expression of C/EBPδ in airway epithelial cells which in turn upregulates PAFR expression. Pneumococci then bind PAFR and invade cells via clathrin coated pits.

There are a few studies about methods of blocking pneumococcal adherence to airway epithelial cells. One such study found that the probiotic, lactobacillus rhamnosus GG (LGG), binds to epithelial cells and prevents pneumococcal adherence. This probiotic had no effect on the release of inflammatory cytokines by the cells¹³. The antibiotic, erythromycin, has also been shown to decrease erythromycin-resistant pneumococcal adherence to airway epithelial cells and delay the disruption of the epithelial layer. Release of interleukin 8 (IL-8) by the cells was not, however, attenuated by the antibiotic¹⁴. S-carboxymethylcysteine (S-CMC), a mucolytic drug, has also been shown to reduce adherence of *S. pneumoniae* to airway epithelial cells. PAFR mRNA and protein expression were also inhibited by this drug¹⁵.

The airway epithelium is the first line of defence against pneumococcal invasion. Alveolar epithelial cells produce, secrete and recycle the alveolar lining fluid which contains cytokines such as TNF- α and IL-6, defensins, surfactant proteins and components of the complement system which are all involved in the innate immune response to microbial infections¹⁶. It has also been shown that tracheal submucosal glands secrete lysozyme which is an antimicrobial¹⁷. A study of airway epithelial cells also showed that infection with pneumococci causes the release of antimicrobial peptides, such as defensins, and the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), which is a pro-inflammatory transcription factor¹⁸. Epithelial cells activate the innate immune response which leads to the recruitment of macrophages to the site of infection. Mice in which the receptor for the immunomodulatory lipid, prostaglandin E₂, was knocked out showed increased bacterial clearance and survival and increased neutrophilia in the lungs. Alveolar macrophages from these mice also showed increased phagocytosis of *S. pneumoniae* and increased tumour necrosis factor α (TNF- α) release¹⁹. Studies of alveolar macrophages also showed that pneumococcal uptake followed by intracellular digestion of pneumococcal peptidoglycan and pneumolysin-dependent sensing by the pattern recognition receptor, nucleotide-binding oligomerisation domain-containing protein 2 (Nod2), leads to the release of chemokines for the recruitment of more macrophages to the site of infection²⁰. It was observed that the release of inflammatory molecules, such as interferons, and the activation of the cytosolic microbial DNA sensing mechanisms was also dependent on the presence of pneumolysin in the cytosol, after pneumococcal phagocytosis by alveolar macrophages²¹. Complement activation provides both antibody dependent and independent protection against pneumococcal infection. The complement system may be activated by the classical, alternative or the lectin pathways^{22,23}. It has been shown that complement proteins, such as C1q, may be able to bind directly to the pneumococcus in an antibody independent manner.

These interactions have been shown to be mediated by bacterial surface proteins and to facilitate pneumococcal adherence and invasion of airway epithelial cells²⁴. Mouse knockout studies have also implicated other molecules such as the receptor for IL-1 and the scavenger receptor, CD36, in immunity against pneumococcal infection. Mice deficient in the IL-1 receptor had higher mortality rates and bacterial burdens although neutrophil recruitment was not affected. In these mice activation of fibrinogen genes involved in coagulation, which is believed to confer protection against invasive pneumococcal disease, was not observed²⁵. CD36 negative mice showed increased inflammation but decreased bacterial clearance and phagocytosis of pneumococci by alveolar macrophages. CD36 has been shown to be expressed by alveolar macrophages and respiratory epithelial cells²⁶.

Pneumococcal persistence in the respiratory epithelium and activation of the immune response leads to disruption of the epithelial barrier and tissue damage. This allows the pneumococci to translocate across the epithelial barrier into the blood stream and cause invasive disease. It has been shown that the PAFR-pneumococcus complex is endocytosed in a clathrin-dependent manner. β arrestin 1 is involved in targeting this endocytosed vacuole away from the lysosome and back to the cell surface⁹. This is a suggested mechanism for pneumococcal transcytosis across the epithelial barrier. Hypoxia inducible factor has also been implicated in pneumococcal transcytosis due to its role in inducing and regulating PAFR expression by binding to the promoter region of the PAFR gene²⁷.

1.1.2 Rhinovirus infection

Virus associated pneumonia is common in both adults and children, with rhinoviruses being one of the leading causative agents, alongside influenza and respiratory syncytial virus^{28,29}. Secondary infections by bacteria such as *S. pneumoniae* exacerbate respiratory symptoms and pneumonia³⁰. The role of

rhinoviruses in infection will be reviewed first followed by a review of the interactions between viruses and bacteria when they co-infect a host.

Rhinoviruses (RVs) are positive single stranded RNA viruses of the picornavirus family. They cause the common cold and have been implicated in exacerbations of asthma and COPD³¹. Intracellular adhesion molecule 1 (ICAM-1), which is an integral membrane protein, has been shown to be an entry receptor for RVs in human cells³². In respiratory epithelial cells it has been shown that RV infection upregulates the expression of ICAM-1 in an NF- κ B-dependent manner. NF- κ B binds to the promoter region of the ICAM-1 gene and induces its expression after RV infection, as shown in figure 2³³. Higher protein and mRNA expression levels of ICAM-1 and NF- κ B have been observed in the nasal mucosa of human subjects with rhinitis³⁴. It has also been shown that virus replication, viral load, the intracellular anti-viral response and the release of cytokines such as IL-8 and IL-1 α are higher in human bronchial epithelial cells compared to human nasal epithelial cells³⁵.

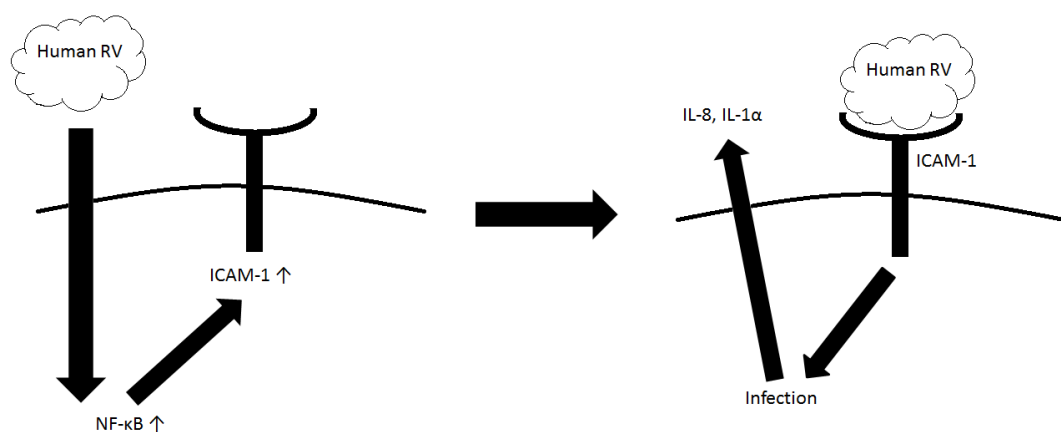


Figure 2: Rhinoviruses infect human cells via ICAM-1. RVs induce NF- κ B expression, which in turn upregulates ICAM-1 expression. ICAM-1 is a known entry receptor for RVs in human cells. RV infection of airway epithelial cells has been shown to cause release of pro-inflammatory cytokines such as IL-8 and IL-1 α .

A few studies have shown that antibiotics and mucolytic drugs may be used to treat rhinovirus infections. A study in bronchial epithelial cells showed that treatment with the macrolide, azithromycin, upregulates the antiviral interferon response and other antiviral genes. Viral replication and release was inhibited by the drug³⁶. Another study showed that levofloxacin reduces viral load, cytokine release, ICAM-1 expression and NF-κB activation in human tracheal cells. A similar study showed similar effects of the mucolytic drug, carbocysteine, in tracheal cells^{37,38}. Iota carrageenan, a sulphated galactose polymer derived from Rhodophyceae seaweed has been shown to inhibit RV growth, replication and entry into nasal epithelial cells *in vitro*. The compound has been approved for use as a nasal spray after studies in both adults and children showed that it reduces viral replication and the release of pro-inflammatory cytokines such as IL-8³⁹⁻⁴¹.

1.1.3 Viral and secondary bacterial co-infections

Secondary bacterial infections are a common occurrence following viral infection. They may exacerbate chronic respiratory conditions, such as asthma and COPD, which may lead to fatalities due to pneumonia^{28,29}.

In a study of pharyngeal cells co-infected with respiratory syncytial virus (RSV) and pneumococci, it was found that adherent pneumococci had increased transcriptional levels of genes encoding adhesins such as PspA and genes involved in choline uptake and incorporation⁴². These data have implications for bacterial adherence to respiratory epithelial cells, as alluded to earlier. An *in vitro* study using human pharyngeal cells showed that exposure to polyinosinic:polycytidylic acid (poly I:C), which is structurally similar to double stranded RNA contained in many viruses and hence stimulates the anti-viral response, increased PAFR mRNA and protein expression. Pneumococcal adherence to these cells was also increased and was attenuated using a PAFR antagonist. In this study it was also observed that the PAFR antagonist did not affect basal levels of adherence of *S. pneumoniae* to the

cells⁴³. In a study in mice it was observed that treatment with poly I: C followed by pneumococcal infection reduced bacterial clearance and increased mortality. These effects were dependent on the activation of antiviral mechanisms such as retinoic acid-inducible gene 1 (RIG-1) signalling, which is involved in the recognition of viral nucleic acid, and interferon production. In mice deficient in type I interferon signalling the impairment of bacterial clearance was not observed⁴⁴. In an *in vitro* study of lower airway epithelial cells it was observed that infection with viruses such as influenza and RSV promoted adherence of *H. influenzae* and *S. pneumoniae*. Differential regulation of human adhesion molecules such as PAFR was seen by the different viruses used in the different cell types tested. Basal levels of these molecules also differed by cell type⁴⁵. A few mouse studies have shown a synergistic effect of influenza infection followed by pneumococcal infection on weight loss, survival, airway repair responses and apoptosis of airway epithelial cells and alveolar macrophages^{46,47}. Additionally, in influenza infected PAFR deficient mice or mice treated with a PAFR antagonist reduced lethality, lung injury and neutrophil recruitment was observed. PAFR expression was higher in mice co-infected with influenza and *S. pneumoniae* compared to mice infected with either influenza or *S. pneumoniae* alone^{48,49}. In mice and *in vitro* it has also been shown that co-infection of rhinovirus and *H. influenzae* causes reduced chemokine production by lung epithelial cells and reduced neutrophil recruitment to the lungs⁵⁰. An *in vitro* study also showed increased pneumococcal adherence to rhinovirus infected epithelial cells. An increase in PAFR expression and NF- κ B activation was also observed. The increased adherence of pneumococci was attenuated by both a PAFR blocker and an antibody against ICAM-1, which is an entry receptor for rhinoviruses³⁰. RSV infection increased PAFR expression in lower airway epithelial cells. Treatment of cells infected with the virus with fosfomycin, an antimicrobial and immunomodulatory agent, attenuated this increased PAFR expression and pneumococcal adherence to the cells⁵¹.

In the following sections the different types of air pollution and their effects on the respiratory system and respiratory infections will be reviewed, with a focus on pneumococcal pneumonia.

1.1.4 Types of airborne environmental pollutants

Both indoor and outdoor air pollution are major health risks in both the developing and developed world. Sources of pollution may broadly be divided into fuel combustion, tobacco smoking and occupational. Pollutants may be particulate or gaseous in nature. Further, exposures may be in outdoor or indoor settings. In the following sections, the evidence that exposure to the different types of air pollution increases vulnerability to respiratory disease and pneumococcal infection will be reviewed.

1.1.5 Indoor and outdoor air pollution caused by fuel combustion

Air pollution caused by fuel combustion may be from a variety of sources. Typical sources of indoor air pollution include the combustion of biomass or wood. Outdoor air pollution may be in the form of diesel exhaust particles, carbon black, nanoparticles or miscellaneous metallic particulate matter with an aerodynamic diameter $< 10\mu\text{m}$ (PM_{10}).

1.1.6 Air pollution and respiratory disease: epidemiology

Globally, billions of people rely on biomass such as wood charcoal and dung for fuel. Biomass smoke contains a number of pollutants such as particulate matter, carbon monoxide and sulphur oxides. Exposure to indoor air pollution has been implicated in respiratory illnesses such as acute respiratory infections and chronic obstructive pulmonary disease (COPD) and maternal exposure is a risk factor for low birth weight. The National Ambient Air Quality Standards of the U.S. Environmental Protection Agency recommends a 24 hour average exposure to PM_{10} of $150\mu\text{g}/\text{m}^3$, which is not to be exceeded more than once a year over a 3 year

period, whereas exposures may be in the range of 200-5,000 $\mu\text{g}/\text{m}^3$ in a household using biomass as a primary source of energy⁵². The European Directive limit of exposure, which the UK must comply with, is a mean of 50 $\mu\text{g}/\text{m}^3$ over a 24 hour period which is not to be exceeded more than 35 times a year⁵³. According to estimates for the period 2000-2003, presented by the WHO, 19% of deaths in children aged < 5 years were due to acute respiratory infections, for which exposure to indoor air pollution is a major risk factor⁵⁴. Recent WHO estimates show that 4.3 million people a year die from exposure to indoor air pollution (accessed 13th January 2015). In a study conducted in Pakistan it was shown that exposure to biomass smoke is associated with chronic bronchitis in women. However, as the smoking status of the women in the study was unknown smoking was a confounder⁵⁵. Additionally, in a study of 271 female children under the age of 5 years in Gambia it was found that carriage of the child on the mother's back during cooking increased the risk of lower respiratory tract infections⁵⁶. A study of 244 children aged 1-3 years in Zimbabwe revealed an association between indoor exposure to wood smoke and risk of infection⁵⁷. Higher exposure to domestic smoke was associated with moderate to severe acute respiratory illness in children less than 2 years of age in Nepal⁵⁸.

COPD, which is a growing global epidemic, is exacerbated by viral and bacterial infections. Rhinoviruses, *H. influenzae* and *S. pneumoniae* are frequently associated with COPD exacerbations^{59,60}. The disease was previously considered a disease of the developed world caused by chronic cigarette smoking. However, epidemiological evidence shows an association between exposure to smoke produced by the burning of biomass fuels and the risk of developing COPD. A systemic review conducted in 2009 showed an association after adjusting for confounding factors such as cigarette smoking⁶¹. A study conducted in Spain also showed independent associations of wood and charcoal smoke exposures with

COPD. This study was also adjusted for confounding factors such as smoking. Similar studies have also been conducted in Mexico and in areas of low socio-economic status in Colombia^{62,63}. In a study conducted in California between 2000 and 2003 an association between acute exposure to particulate matter (PM), from the combustion of fuels such as gasoline and diesel, and hospitalisation for respiratory illnesses such as pneumonia and bronchitis has been shown in children < 5 years of age and < 19 years of age⁶⁴. Additionally, in a European study of 10 birth cohorts a significant association between parent reports of physician diagnosed pneumonia and air pollution was found in children aged 12-36 months⁶⁵.

1.1.7 Air pollution and respiratory disease: mechanisms

In vitro studies have shown that lower airway epithelial cells such as alveolar cells take up particulate matter into vesicles in the cytoplasm⁶⁶. Genetic profiling of bronchial epithelial cells, *in vitro*, showed an upregulation of genes involved in the oxidative stress response after exposure to PM⁶⁷. Further, when these cells were exposed to PM there was an increase in intracellular reactive oxygen species (ROS) which in turn lead to an increase in transforming growth factor β 1 (TGF- β 1) and fibronectin produced by the cells. Both these molecules play key roles in wound repair mechanisms which are aberrant in pulmonary fibrosis. In this system the use of an anti-oxidant, N-acetyl cysteine (NAC), attenuated ROS production and thus the production of TGF- β 1 and fibronectin by these cells⁶⁸. NAC replenishes stores of the biologic anti-oxidant, glutathione. In a study of primary bronchial epithelial cells exposure to PM induced phosphorylation of protein kinases, such as extracellular signal-regulated kinase (ERK), which regulate the inflammatory response. Further, a glutathione transferase gene which is involved in the oxidative stress response, was knocked down *in vitro*, which significantly increased the production of PM stimulated inflammatory cytokines IL-8 and IL-1 β . The use of NAC in this system inhibited the abovementioned phosphorylation of the protein kinases and subsequently the

production of IL-8 and IL-1 β ⁶⁹. A study in alveolar epithelial cells confirmed the role of these protein kinases in the anti-oxidative stress response and showed that exposure to PM induces the nuclear factor erythroid-2-related factor 2 (Nrf2) signalling pathway which regulates intracellular defence mechanisms against oxidative stress⁷⁰. Exposure to PM has also been shown to inhibit cell proliferation and to induce the production of the pro-inflammatory cytokine, granulocyte macrophage-colony stimulating factor (GM-CSF), in bronchial epithelial cells⁷¹. Other *in vitro* studies have also shown an increase in the production of enzymes such as matrix metalloproteinase 1 (MMP-1) by bronchial epithelial cells and an increase in apoptotic tendency regulated by B-cell lymphoma 2 (Bcl2) and c-Myc, after exposure to PM^{72,73}. MMP-1 is involved in the degradation of the airway extra cellular matrix and has been implicated in chronic respiratory diseases such as COPD and asthma.

The activation of the inflammatory response by airway epithelial cells following PM endocytosis leads to the recruitment of inflammatory cells such as monocytes, macrophages and dendritic cells to the sites of deposition, which then perpetuate the immune response. There are a few studies which have looked at the PM-mediated interactions between lower airway epithelial cells and monocytes or macrophages. These *in vitro* studies have shown that exposure to PM increases the production of inflammatory cytokines such as IL-8 and TNF- α in these systems^{14–17}. Additionally, it has been shown that exposure to wood smoke particles significantly increased the production of inflammatory cytokines such as interleukin-6 (IL-6), IL-8 and tumour necrosis factor α (TNF- α) in an airway epithelial cell/monocyte co-culture system. Lactate dehydrogenase (LDH) release from damaged cells was also significantly increased. Epithelial cells and resident macrophages in the alveoli are the primary cellular targets for particle deposition in the lungs and monocytes, which are the precursors of macrophages, accumulate in the alveoli during inflammation

and assist with particle clearance. Particle deposition in the alveolar region is highly uneven and clearance is slow. DNA damage, such as strand breaks, was also observed in both types of cells *in vitro*^{77,78}. Further, it has been observed that exposure of monocytes/macrophages to PM stimulates calcium signalling mediated TNF- α release and inhibits the phagocytic function of these cells^{79,80}. Phagocytosis is an important defence mechanism against bacterial infection. PM exposure enhances dendritic cell maturation, *in vitro*, which is a critical process in activating and mediating the T helper cell response which is part of the adaptive immune system⁸¹. Additionally, a study in mice showed that acute exposure to PM causes local and systemic inflammation and skews the T helper cell response⁸². Exposure of mice to PM caused an increase in vascular endothelial growth factor (VEGF) levels in the lungs which in turn led to protein leakage into the lungs and neutrophil infiltration, both of which cause lung inflammation and injury⁸³. VEGF is a vascular permeability factor. Another *in vivo* study showed increased airway neutrophilia in PM exposed mice⁸⁴.

1.1.8 Air pollution and infection: mechanisms

There are a number of studies which show an association between exposure to PM and incidence or severity of respiratory infections. *In vitro* studies in nasal epithelial cells showed increased replication of the virus which causes the common cold, RV, after exposure to PM. The release of inflammatory cytokines such as interferon γ (IFN- γ), IL-1 β and IL-6 was also increased in these cells⁸⁵. Another study in lower airway epithelial cells showed increased invasion of these cells by *Streptococcus pneumoniae* (*S. pneumoniae*) after exposure of the cells to PM. This increased invasion was attenuated by treatment of the cells with the anti-oxidant, NAC, and by treatment with a PAFR blocker. PAFR is co-opted by pneumococci for invasion of human cells⁸⁶. PM exposed mice had a greater load of pneumococci in the lungs with alveolar macrophages displaying reduced lymphocyte activation activity⁸⁷.

Another study showed that PM is a source of bioavailable iron for bacteria and that bacterial growth increased in the presence of PM. The study also showed that airway epithelial cells exposed to PM had decreased antimicrobial activity and that mice exposed to PM and infected with *Pseudomonas aeruginosa* (*P. aeruginosa*) showed decreased bacterial clearance⁸⁸. Mice exposed to PM during an influenza infection had increased viral titres, increased numbers of neutrophils and increased levels of inflammatory cytokines in the lungs⁸⁹.

1.1.9 Tobacco smoking and respiratory disease: epidemiology

Tobacco smoking is a leading cause of preventable deaths. Tobacco smoking and exposure to second hand smoke increase risk of adverse health outcomes such as heart disease, cancer and respiratory illnesses such as COPD in adults and pneumonia and bronchitis in both adults and children⁹⁰. Smoking also affects the immune system and fertility⁹¹. COPD is a chronic respiratory disease, the main cause of which is chronic smoking, characterised by inflammation of the bronchi, damage to the small airways and alveolar sacs and restricted airflow. The disease is exacerbated by bacterial and viral infections which may also be fatal. According to the WHO approximately 3 million people die every year as a consequence of having COPD^{92,93}.

There is an association between cigarette smoking or passive exposure to cigarette smoke and respiratory infections. In Vietnam it was shown that over a 12 month period the number of hospital admissions of children < 5 years of age who were exposed to cigarette smoke was significantly higher than those unexposed⁹⁴. Additionally, in a study of 500 children under the age of 5 years in Gambia an association between father's smoking and risk of developing lower respiratory tract infections was found⁵⁶. In a review of reports published between 1966 and 1995 it was found that children with a smoking parent were at twice the risk of developing a severe respiratory tract illness requiring hospitalisation, in early life⁹⁵.

In adults, studies in India have shown a dose response relationship between tobacco smoking and pulmonary tuberculosis in 85 men aged 20-50 years and an association between risk of tuberculosis and smoking in 93945 individuals aged over 15 years^{96,97}. Another study in India showed a relationship between smoking and underlying conditions such as COPD and community acquired pneumonia in 70 patients⁹⁸. In a study of non-elderly immunocompetent adults aged 18 to 64 years in the US, smoking was found to be a strong independent risk factor for pneumococcal disease. Increased risk was also associated with passive smoking⁹⁹.

1.1.10 Tobacco smoking and respiratory disease: mechanisms

In vitro studies in nasal epithelial cells have shown that exposure to cigarette smoke extract (CSE) alters the immune response of these cells, with short term exposure suppressing the immune response and long term exposure triggering a pro-inflammatory response¹⁰⁰. Exposure of lower airway epithelial cells, such as alveolar and bronchial epithelial cells, to tobacco smoke or cigarette smoke condensate (CSC) showed an increase in the release of inflammatory cytokines such as IL-6, IL-8 and IL-1 β in a mitogen activated protein (MAP) kinase and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) dependent manner^{101,102}. MAP kinases are part of the signalling pathway which leads to the activation of NF- κ B which is a ubiquitous pro-inflammatory transcription factor.

Cigarette smoke exposure of murine lower airway epithelial cells from Nrf2 deficient mice caused greater injury compared to cells from wild type mice. This effect was reversed by the use of the anti-oxidant NAC¹⁰³. As mentioned earlier, Nrf2 is involved in oxidative stress defence mechanisms and NAC acts by restoring glutathione, which is a biological anti-oxidant, levels. Another anti-oxidant, vitamin C, was used in alveolar epithelial cells and in guinea pigs to show a reduction in NF- κ B activation after exposure to cigarette smoke¹⁰⁴. An *in vitro* study of alveolar epithelial cells showed an increase in aldehyde and oxidant induced apoptosis after

exposure to CSE¹⁰⁵. Further, exposure of alveolar epithelial cells and mice to cigarette smoke induced cellular senescence in the lung epithelium¹⁰⁶. It also induced endoplasmic reticulum (ER) stress caused by reduced activity of the ubiquitin-proteasome pathway which leads to the accumulation of damaged proteins^{107,108}. Cellular senescence and ER stress cause epithelial cell death, disruption of the epithelial barrier and prevention of proliferation for repair, which all contribute to the development of chronic lung diseases such as COPD. Expression of MER tyrosine kinase (MERTK), a transmembrane tyrosine kinase receptor involved in the phagocytosis of apoptotic cells, in alveolar macrophages was also observed to be significantly higher in smokers compared to non-smokers¹⁰⁹. This may reflect the increased apoptosis of lower airway epithelial cells observed *in vitro*. Additionally, an increase in oxidative stress, DNA fragmentation and apoptosis was observed in lung fibroblasts, which are involved in airway remodelling, after exposure to CSE *in vitro*¹¹⁰. *In vitro* chemotaxis assays demonstrated that exposure of alveolar epithelial cells to CSE stimulated chemotactic activity in monocytes and neutrophils¹¹¹. This suggests that exposure to CSE modulates the recruitment of inflammatory cells to the lungs. Indeed, repeated exposure of guinea pigs to cigarette smoke for 14 days caused significant neutrophilia in the lungs¹¹². In addition, exposure to CSE increases the release of platelet activating factor (PAF) by endothelial cells. PAFR promotes trans-endothelial migration of inflammatory cells into the airways. In this study a PAFR receptor (PAFR) blocker, CV-3988 which mimics PAF, attenuated the adherence of leucocytes to the endothelial cells⁹¹.

1.1.11 Chronic smoking and COPD: mechanisms

Bronchial epithelial cells from COPD patients have been shown to constitutively release higher levels of IL-6 and IL-8. Stimulation of these cells with lipopolysaccharide (LPS), which is a bacterial endotoxin, from *P. aeruginosa* has

been shown to attenuate inflammatory responses such as IL-8 release and NF- κ B activation¹¹³. These findings suggest that the immune response to bacterial infections is suppressed in COPD patients, which is in line with observations of increased susceptibility to infections in these patients. The bronchial epithelium from these patients has also been shown to express lower levels of the anti-oxidant gene, catalase, compared to non-smokers and smokers without COPD. Catalase blocks the activation of NF- κ B and decreased levels in mice has been shown to cause increased cell death¹¹⁴. Smoking has also been shown to reduce the phagocytic function of alveolar macrophages, which is a key lung defence mechanism, in both smokers and COPD patients. It was also observed that the phagocytic function of these macrophages is better in COPD patients who are ex-smokers compared to those who are current smokers¹¹⁵. Goblet cell metaplasia, which contributes to airway obstruction and causes mucus hypersecretion, has been shown to be higher in the trachea in a guinea pig model of COPD. Increased numbers of neutrophils were also seen in the broncho-alveolar lavage fluid (BALF). A PAFR antagonist attenuated both the increased goblet cell metaplasia and the increased neutrophilia in this model¹¹⁶.

Rhinovirus infections are a common cause of COPD exacerbations and secondary bacterial infections are commonly reported. In a human model of experimental rhinovirus infection it was found that a significantly higher proportion of COPD patients developed secondary bacterial infections compared to healthy smokers and non-smoking controls. The COPD patients that developed secondary bacterial infections had lower sputum levels of antimicrobial peptides which inversely corresponded with bacterial load. They also had higher viral loads⁶⁰.

1.1.12 Cigarette smoking and infection: mechanisms

A study showed that lipopolysaccharide (LPS), which is a bacterial endotoxin and an inflammatory stimulus, is a component of cigarette smoke¹¹⁷. In a study of the

oral microflora of smokers it was found that the growth of *P. aeruginosa* is not inhibited by smoking but the growth of *S. pneumoniae* and *Haemophilus influenzae* (*H. influenzae*) is¹¹⁸. *In vitro* studies in buccal cells from smokers and non-smokers showed increased adhesion of bacteria such as *S. pneumoniae* and *H. influenzae* to cells from smokers^{119,120}. This suggests that although the growth of some bacteria may be inhibited by exposure to cigarette smoke, exposure to the smoke makes airway epithelial cells more susceptible to invasion by these bacteria. Bacterial adherence to epithelial cells is the first step in the colonisation process. Other *in vitro* studies have shown that exposure of upper airway epithelial cells to CSE or CSC followed by infection by bacteria such as *Staphylococcus aureus* (*S. aureus*) and *H. influenzae* decreases production of inflammatory cytokines such as IL-8 and IL-6 and activation of the pro-inflammatory transcription factor NF- κ B^{121,122}. It has also been shown that exposure of lower airway epithelial cells to CSE, *in vitro*, increases vulnerability to pneumococcal infection. This vulnerability was attenuated by a PAFR blocker in this study. This study also showed that PAFR expression was increased in the lungs of mice exposed to cigarette smoke. Bronchial epithelial PAFR protein expression was also raised in smokers' lungs¹²³.

In a study of current and former smokers with acute pneumonia it was observed that pharyngeal wash fluid and sputum had reduced levels of antibacterial peptides such as human β defensin 2 (hBD-2)¹²⁴. This suggests that cigarette smoking suppresses the pulmonary innate immune system. A mouse study showed that exposure to cigarette smoke and infection with *Mycoplasma pneumoniae* (*M. pneumoniae*) caused an increase in DNA oxidation in the lungs. This increase was also observed in lung epithelial cells *in vitro*¹²⁵. Buccal cells of rats chronically exposed to cigarette smoke, *in vivo*, were more susceptible to pneumococcal adherence¹²⁶. Additionally, mice chronically exposed to cigarette smoke and infected with *S. pneumoniae* had greater lung infiltration by macrophages and neutrophils and higher levels of

inflammatory molecules such as TNF- α in the lungs. They also displayed more weight loss. However, in this model, bacterial burden in mice exposed and unexposed to cigarette smoke was comparable¹²⁷. In a more susceptible mouse pneumococcal model it was observed that bacterial burdens were higher in cigarette smoke exposed mice compared to the controls. In this study it was also shown that alveolar macrophages exposed to CSE *in vitro* and infected with *S. pneumoniae* had reduced phagocytic activity¹²⁸.

Cigarette smoking not only alters host defence systems, as outlined above, but also affects the pathogens that cause respiratory infections. Exposure of *S. pneumoniae* to CSC increased its ability to form biofilms and the pore forming activity of a major toxin, pneumolysin. Biofilm formation insulates pathogens from host defence systems, which prolongs bacterial persistence¹²⁹. Other studies have also shown alterations in expression of virulence genes in bacteria such as *Porphyromonas gingivalis* (*P. gingivalis*) after exposure to cigarette smoke¹³⁰.

1.1.13 Occupational exposures to airborne particulate matter

Workers across a number of industries worldwide are chronically exposed to hazardous agents such as biological and chemical agents, and radiation in occupational settings. The National Institute for Occupational Safety and Health, which is part of the Centre for Disease Control and Prevention (USA), has identified exposure to aerosols such as dust, diesel emissions, nanotechnology and metal fumes as a significant area of concern. Additionally, a recent systematic literature review revealed an association between occupational exposure to vapours, gas, dust and fumes and the risk of COPD, both within and across different industry groups¹³¹.

The focus of this thesis is metal fumes and more specifically, welding fumes. Welding (figure 3) has previously been included as a target occupation for the

Disease Reduction Programme (DRP) because of severe ill-health effects seen in welders. The DRP is part of the work done by the Health and Safety Executive which is the independent watchdog for occupational health and safety in the UK.



Figure 3: Welding is a sculptural or fabrication process by which two metal work pieces are joined. An electric arc is often used as a source of energy for the process. The image was adapted from the National Careers Service website (UK) on 9th February 2014.

1.1.14 Occupational exposure to metal fumes

Epidemiology: Several million people worldwide are currently estimated to be full time occupational welders. Occupational exposure to welding fume is associated with adverse respiratory outcomes such as lobar pneumonia, asthma and lung cancer. A number of epidemiological studies conducted in countries such as the UK, US and Sweden have shown an association between welding and a decline in lung function. Some of these studies suggest a greater effect in welders who smoke. It is currently estimated that the total welding fume dose that welders are exposed to during their working lives is approximately $80\text{mg/m}^3 \cdot \text{years}^{132,133}$. Welding is the fusion of metals at a high temperature generated using a gas metal arc (GMA). Mild steel (MS) wires are the most commonly used in welding but the use of stainless steel (SS) wires protects the weld from corrosion. Fume generated by welding is a mixture of compounds of metals such as iron, chromium and manganese.

Mechanisms: In an *in vivo* study in mice chronically exposed to welding fumes by aspiration, the lung transcriptome was analysed and it was observed that genes implicated in immunological disorders, cancer and cell death were dysregulated¹³⁴. In another study in cynomolgus monkeys, which have a highly homologous genome to humans, genetic profiling of the lungs revealed that chronic inhalation exposure to welding fume caused the dysregulation of genes involved in inflammation, cancer and the cell cycle. Histopathological analysis of the lungs showed deposition of welding fume in the airways¹³⁵. This was similar to a rat study in which rats were chronically exposed to stainless steel welding fumes. Inflammatory cell influx and pulmonary injury were observed in these rats. Further, iron, chromium and nickel complexes were observed in the lungs and in alveolar macrophages¹³⁶. A histopathological study in welders also showed weld generated particulate matter in the lungs¹³⁷. In a mouse study it was observed that chronic exposure to welding fumes by aspiration caused a significant increase in lung tumour multiplicity¹³⁸. Additionally, in rats a higher number of macrophages and neutrophils and an increased amount of TNF- α was observed in the bronchoalveolar fluid (BALF)¹³⁹. In another *in vivo* study DNA damage in the lung was seen in rats chronically exposed to welding fumes by inhalation¹⁴⁰. A study in alveolar epithelial cells showed an increase in IL-8 production and the production of ROS in these cells after exposure to welding fume *in vitro*. A depletion of cellular glutathione levels was also observed in this system¹⁴¹.

1.1.15 Welding and pneumonia

Epidemiology: In a study of occupational mortality in England and Wales in welders aged 15-64 years it was found that mortality from pneumonia was higher in 1959-1963 and in 1970-1970. Additionally, mortality from pneumococcal and lobar pneumonia was increased in 1979-1980 and in 1982-1990. No excess in deaths was seen in men aged above the retirement age (65 years)¹⁴². In a case control

study conducted in 1996-1999 in men aged 20-64 years it was observed that pneumonia was associated with exposure to welding fume within a year of hospitalisation in 525 welders compared to 1,122 controls⁴. Finally, in a study of deaths due to pneumonia in 1991-2000 in men aged 16-74 years it was found that observed deaths, due to lobar pneumonia, were significantly higher than expected deaths. However, this was not the case in men of older ages¹⁴³. Taken together, the data suggest that exposure to welding fume reversibly increases susceptibility to pneumonia. A retrospective chart review of invasive pneumococcal disease in 2000-2004 in men aged 18-65 years in Canada showed a 2.7 fold greater incidence of disease in welders compared to the general population. 15 of the 18 welders in the study were current or former smokers, making the rate of smoking in welders higher when compared the general population. This suggests that cigarette smoking is highly prevalent amongst welders and hence exposure to cigarette smoke is an additional risk factor for these individuals¹⁴⁴.

Mechanisms: Very little is known about the mechanisms that cause this increased susceptibility of welders to pneumonia. In rats exposed to welding fume and infected with *Listeria monocytogenes* (*L. monocytogenes*) a higher bacterial burden was observed^{145,146}.

1.1.16 Exposure to inhalable particulate matter and viral/bacterial interactions during co-infection: mechanisms

There is very little known about how exposure to pollutants may affect viral/bacterial interactions in diseases such as pneumonia. A study in rat lung epithelial cells showed that exposure to diesel exhaust particles increased mRNA expression of ICAM-1, PAFR and a marker of oxidative stress, heme oxidase-1¹⁴⁷. These data suggest that exposure to PM may increase susceptibility to airway epithelial cells to viral and bacterial infections. Exposure of RV infected bronchial epithelial cells to CSE caused a suppression of antiviral response genes such as melanoma

differentiation-associated protein 5 (MDA5) and RIG-1 in those cells. Both molecules are cytoplasmic RNA helicases which sense double stranded RNA. Interferon production, which is also part of the antiviral response, was not inhibited by CSE exposure in these cells¹⁴⁸.

1.2 Summary

Pneumonia is one of the leading causes of deaths worldwide. The major causative agent of bacterial pneumonia is *S. pneumoniae*. Exposure to airborne particulate matter, such as in occupational settings and cigarette smoking, is a major risk factor for respiratory diseases such as pneumonia and COPD. Infection with viruses such as rhinoviruses increases susceptibility to secondary bacterial infections and exacerbates pneumonia and COPD.

As evidenced by a review of the literature above, very little is known about the molecular mechanisms involved in causing susceptibility to pneumococcal infections in welders. Currently in the UK welders are not routinely offered the pneumococcal vaccine. Identifying the mechanisms involved is the first step towards developing therapies for targeting pneumococcal pneumonia in welders.

1.3 Aims

The aims of this thesis are to:

- Determine the effect of welding fumes on pneumococcal and rhinoviral and secondary pneumococcal infections in the lower airway epithelium.
- Establish whether PAFR expression is elevated in the bronchial and alveolar epithelium of smokers with COPD.

1.4 Hypotheses

1. Exposure to metal fumes generated during welding increases vulnerability to:
 - Pneumococcal infections in a PAFR and oxidative stress dependant manner.
 - Rhinoviral infections and secondary pneumococcal infections.
2. COPD patients, who are highly susceptible to both viral and bacterial infections, have increased PAFR expression in the lung epithelium.

Chapter 2: Methods

2.1 Cell culture

A549s are type II pneumocytes isolated from a carcinoma. For this study the cells were obtained from Sigma Aldrich (UK). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% L-glutamine (G)/penicillin-streptomycin (P/S) (Lonza, Switzerland). Passage number was <20. Experiments were conducted using incomplete DMEM without either FBS, G or P/S.

BEAS-2B cells are bronchial epithelial cells that were isolated from a healthy individual and transformed using Simian virus 40. For this study the cells were kindly donated by Dr Nicolas Mercardo (National Heart and Lung Institute, Imperial College London, UK). The cells were initially grown in DMEM as above but it was found that during experiments in incomplete media the cells lifted off the plates. Thereafter, cells were grown in Roswell Park Memorial Institute-1640 media containing HEPES (RPMI)(Life Technologies, UK) 10% FBS and 1% G, P/S. Passage number was <20. Experiments were conducted using RPMI containing 2% FBS and plates were coated with collagen type I from calf skin (Sigma Aldrich, Poole, UK).

RPMI2650 cells are nasal epithelial cells isolated from a squamous cell carcinoma from the nasal septum. For this study the cells were obtained from LGC Standards (UK). Cells were grown in DMEM with 10% FBS and 1% PSG. Passage number was <20 for all experiments. Experiments were conducted using incomplete media as described for the A549 cells. All cells were grown at 37°C and supplemented with 5% CO₂.

The virulent type 2 *S. pneumoniae* encapsulated strain D39 (NCTC 7466) was purchased from the National Collection of Type Cultures (Central Public Health Laboratory, UK) and grown in liquid culture brain heart infusion (BHI) broth (Oxoid,

UK) in a water bath, set to 37°C, to mid-logarithmic phase (optical density₆₀₀=0.4-0.6) prior to use *in vitro*.

To determine the OD₆₀₀ at which the bacteria are in mid-log phase, bacterial stocks, stored at -80°C in BHI containing 10% glycerol, were grown over a period of 4 hours and the OD₆₀₀ measured every 30 minutes. Over the OD₆₀₀ range of 0.3 to 0.7, the change in OD₆₀₀ is exponential. The concentration of pneumococci over this range was determined using quantitative bacterial culture and is approximately 10⁹ CFU/ml. Quantitative bacterial culture was carried out using BHI agar plates, containing 5% horse blood, incubated overnight at 37°C and supplemented with 5% CO₂.

2.2 Weld fume collection and analysis

Samples of mild steel and stainless steel welding fumes for use *in vitro* and in the intranasal inoculation model *in vivo*, as detailed later in this chapter, were a kind gift from Geoff Melton (The Welding Institute, UK). Fume samples were captured in accordance with BS EN ISO 15011-1:2009. 'Health and safety in welding and allied processes. Laboratory method for sampling fume and gases. Determination of fume emission rate during arc welding and collection of fume for analysis.'¹⁴⁹ Electrodes were run to produce a weld bead inside the fume collection system. Fume was extracted through the hood on top of the box and collected on a filter paper. This fume sample was then digested in nitric/hydrochloric acid in a high temperature closed vessel microwave assisted dissolution system prior to analysis by Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES). Both samples are from manual metal arc (MMA) welding electrodes. Metal analysis of the samples showed that the major components of the stainless steel fume were potassium, silicon, chromium, manganese and iron. The major components of the mild steel fume were potassium, calcium, manganese, magnesium and iron. The compositions of the two types of welding fumes provided by Geoff Melton are shown

in Table 1. The compositions were supplied by Geoff Melton along with the WF samples.

	Stainless Steel (% of total weight of sample)	Mild Steel (% of total weight of sample)
Aluminium	0.7	0.3
Barium	<0.1	<0.1
Bismuth	<0.1	<0.1
Calcium	0.7	8.9
Cobalt	<0.1	<0.1
Chromium	4.1	<0.1
Copper	<0.1	<0.1
Iron	3.9	12.4
Potassium	27.4	23.6
Lithium	<0.1	0.4
Magnesium	0.1	5.3
Manganese	2.7	3.8
Molybdenum	<0.1	<0.1
Sodium	3.0	2.6
Nickel	0.3	<0.1
Lead	0.1	<0.1
Silicon	8.8	2.1
Titanium	1.5	0.6
Vanadium	<0.1	<0.1
Zinc	0.3	0.2
Fluoride ions	17.4	17.9
Chromium (VI)	3.1	<0.1

Table 1: Compositions of the mild steel and stainless steel welding fumes used in the *in vitro* and intranasal inoculation *in vivo* experiments detailed later on in this chapter. Each element is represented as a percentage of the total weight of the sample provided. The welding fumes were suspended in PBS for use in experiments.

The composition of the stainless steel welding fumes used in the inhalation *in vivo* model, detailed later on in this chapter, was kindly provided by Patti Erderly (Centre for Disease Control, USA) and is shown in Table 2. The major components of the welding fume sample were iron, chromium, manganese, nickel and copper. The particle diameters were less than 10µm.

	Stainless Steel (% of total weight of sample)
Iron	57
Chromium	20.2
Manganese	13.8
Nickel	8.8
Copper	0.2
Silicon	Trace
Aluminium	Trace
Vanadium	Trace

Table 2: Compositions of the mild steel and stainless steel welding fumes used in the inhalation *in vivo* experiments detailed later on in this chapter. Each element is represented as a percentage of the total weight of the sample provided.

2.3 Pneumococcal adhesion to lower airway epithelial cells

The final optimised pneumococcal adhesion assay used is outlined later in this section. However, initial work focused on optimising the assay.

In initial experiments, 2×10^5 epithelial cells/ml were seeded on 24 well plates and left overnight to adhere before use. However, it was found that cells seeded at this concentration did not reach 80% confluence overnight. Hence, 4×10^5 cells/ml were grown to confluence overnight on 24 well plates for experiments.

A previous study in the lab assessed pneumococcal adhesion and invasion of lower airway epithelial cells using a multiplicity of infection (MOI) of 2400⁸⁶. Hence, these conditions were used in initial experiments. However, a large amount of variability was seen when using an MOI of 2400.

Thus the MOI used was titrated down to 100. It was found that using a lower MOI produced tighter and more robust data.

After optimisation of the assay, the following protocol was used for further experiments. Cells were exposed to welding fume in suspension in phosphate buffered saline (PBS) made up to the required concentration in incomplete media for 2 hours or overnight, washed with incomplete media and infected with *S. pneumoniae* at a multiplicity of infection (MOI) of 100. Bacteria were allowed to grow for between 2.5 to 3 hours, till they entered the mid-log growth phase, before use in experiments. Optical density was monitored 2 hours onwards. Prior to use in experiments the bacteria were washed 3 times in endotoxin free PBS (Lonza, Switzerland) and made up to $OD_{600}=0.4-0.6$ in PBS. In this OD_{600} range the concentration of the pneumococci is approximately 10^9 colony forming units (CFU)/ml. They were then diluted in incomplete cell culture media to give an MOI of 100 in experiments. Exposure to incomplete media alone, instead of WF, was used as a control. Cells were vigorously washed in incomplete media, detached and

lysed using sterile distilled water. Serial dilutions of the samples were plated on brain heart infusion agar containing 5% horse blood (Oxoid, UK) in order to assess colony forming units (CFU)/ml.

In order to assess the role of PAFR in adhesion, a PAFR blocker, CV-3988^{150,151} (Sigma Aldrich, Poole, UK), was used at a final concentration of 20µM. A concentration of 10µM was used for previous work in the lab because BEAS-2B cells grown in DMEM did not tolerate a higher dose of CV-3988 and started to lift off. However, it was found that changing the culture conditions of these cells by culturing them in RPMI, as described above, allowed the use of a higher dose of CV-3988 which also produced more robust data. Stock solutions of CV-3988 were made up in dimethyl sulfoxide (DMSO). The blocker was added during exposure of the cells to the welding fume.

The effect of N-acetyl cysteine (NAC) (Sigma Aldrich, UK), which is an anti-oxidant, on adhesion was also assessed. NAC has been shown to replenish levels of the biological anti-oxidant, glutathione¹⁵². Stock solutions of NAC were made up in distilled water. NAC was used at a final concentration of 5mM. This dose was titrated down from a dose of 30M which was used in previous work in the lab. NAC was added during the exposure of the cells to the welding fume.

2.4 Viral infection of lower airway epithelial cells

Human rhinovirus subtype A, serotype 16 (RV-16), 2X10⁷ tissue culture infective dose (TCID₅₀/ml), was a kind gift from Michael Edwards (Imperial College London, UK). Infection of A549 and BEAS-2B cells was carried out as described previously³⁶. Briefly, 4X10⁵ cells were grown overnight in 24 well plates. Cells were infected with virus at MOI 1 or exposed to virus inactivation/filtration controls for 1 hour, ± 25µg/ml iota carrageenan⁴¹, in incomplete media. After 1 hour all virus containing media was replaced with incomplete media before proceeding with the

experiment. For iota carrageenan treated cells, 25 µg/ml IC was added back to the cells and left overnight. An adhesion assay was then carried out as described above.

For the filtration control, virus particles were removed from inocula by ultrafiltration through membranes (Amikon, UK) to remove all molecules greater than 30 kDa, performed according to the manufacturer's instructions. For the inactivation control, viruses were inactivated by exposure to UV light at 1200 mJ/cm² for 30 min.

2.5 Analysis of mRNA levels of genes of interest

RNA was extracted from either cells or tissue using the RNeasy kit (Qiagen, UK). First strand cDNA synthesis was carried out using SuperScript VILO MasterMix (Life Technologies, UK). Real time polymerase chain reactions (RT-PCRs) were carried out using TaqMan Gene Expression MasterMix (Life Technologies, UK). mRNA analysis was carried out using relative quantification involving normalisation to the housekeeping gene. Primer/probe sets used: human housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Hs02758991_g1; human PAFR Hs00265399_s1; human ICAM-1 Hs00164931_m1; mouse housekeeping gene β 2 microglobulin Mm00437762_m1; mouse PAFR Mm02621061_m1 (Life Technologies, UK), all primer/probe sets spanned exon-exon boundaries to control for genomic DNA contamination; information about viral RNA primers and probe was taken from a previous study³⁶. All techniques were carried out according to manufacturer's instructions.

2.6 Imaging methods

A549 cells exposed to 275µg/ml mild steel welding fume were imaged by Dr. Ann Wheeler (Queen Mary University of London, UK) using phase contrast microscopy. Images were taken on a time lapse microscope using a 10x objective. The camera used was EXI Blue (Photometrics, UK).

PAFR protein expression, *in vitro*, after exposure to welding fumes was measured using fluorescence microscopy. Images were taken on a Leica MM DM400 epi-fluorescence microscope using a 40x oil objective. The camera used was QIQuick (QImaging, UK). Images were analysed using ImageJ software (Bethesda, Maryland, USA). 4×10^5 cells were grown on coverslips in 24 well plates. After exposure to welding fumes, as described previously, the cells were fixed in ice cold 4% paraformaldehyde for 10mins at room temperature. The cells were washed 3 times with PBS+10% FCS (wash buffer). Mouse anti-human PAFR immunoglobulin 2a (IgG2a) antibody (1:100, CAY160600, Cayman Chemicals, USA) was left on overnight at 4°C. A mouse IgG isotype control was used (Biolegend, UK). The cells were washed 3 times with wash buffer and an Alexa-fluor 488 conjugated goat anti-mouse antibody applied (1:2000, Invitrogen, UK) at room temperature for 30mins under aluminium foil. The cells were washed 3 times with wash buffer and the nuclear stain, 4',6-diamidino-2-phenylindole, dilactate (DAPI), applied (1:1000, Invitrogen, UK) at room temperature for 15mins under aluminium foil. The cells were washed 3 times with wash buffer and the coverslips mounted on glass slides and sealed using transparent nail varnish. The slides were left to air dry under foil for 4 hours and stored overnight at 4°C for analysis. All antibodies and DAPI were made up in wash buffer.

2.7 Immunohistochemistry

PAFR protein expression and localisation was visualised using immunohistochemistry.

Antigen retrieval was carried out on 3µm paraffin wax embedded sections dried down overnight at 60°C. Slides were placed in an ethylenediaminetetraacetic acid (EDTA) buffer of pH8.1 and microwaved at full power for 35 min. The slides were then transferred to a DAKO Autostainer where they were treated with a 3% peroxidase block followed by the Vector R.T.U Vectastain Kit (PK-7200), according

to manufacturer's recommendations. The working dilution of the human anti-PAFR monoclonal antibody CAY160600 (Cayman Chemical, Cambridge Bioscience, UK) was 1:100 or rabbit anti-mouse polyclonal antibody BS1478R (Bioss Inc, USA) was used at 1:100 and the incubation time was 40 min. The signal was visualised using DAKO DAB + Chromogen solution (K3468) applied for 5 min. A Gills haematoxylin nuclei counterstain was used for 2 min and differentiated accordingly. A negative control using tonsil tissue without the anti-PAFR antibody showed no non-specific DAB signal.

The slides were imaged using an MBF stereology microscope and an Optonics camera, using a 10x air objective.

2.8 Analysis of cell viability

Analysis of cell viability was carried out using the lactate dehydrogenase (LDH) assay (Sigma Aldrich, Poole, UK) according to manufacturer's instructions. Membrane integrity of the cells was measured using the LDH release format of the assay. Distilled water was used as a positive control for cell viability.

2.9 *In vivo* work

2.9.1 *Welding fume intranasal inoculation model*

Experiments, approved by UCL Biological Services Ethical Committee and UK Home Office (Project Licence PPL70/6510), were performed according to UK national guidelines for animal use and care, under the UK Home Office licence in accordance with EU Directive 2010/63/EU.

4-6 week old female CD1 mice (Charles River, UK) were used. The mice were inoculated intra-nasally, under anaesthesia (isofluorane) with mild steel welding fume/ 5×10^6 bacteria in 50µl PBS. Control mice received 50µl PBS. The bacterial stock stored at -80°C was defrosted and resuspended in PBS before delivery. The mice were culled using an overdose of pentobarbital, for assessment of CFU in the

blood, lung and bronchial lavage fluid (BALF) and for cell count and cytospin analysis of the BALF. PAFR mRNA expression levels in the lung were also assessed.

Intervention regimes for this model are shown in Table 3.

Table 3: programme of intervention for instillation mouse model: (A) acute exposure to WF; (B) chronic exposure to WF; (C) for immunohistochemistry analysis.

A: acute exposure to WF; total dose of WF=600µg/mouse

Day	Intervention
1	WF/PBS
2	Bacteria
3	Cull

B: chronic exposure to WF; total low dose of WF=600µg/mouse

Day	Intervention
1	WF/PBS
3	WF/PBS
5	WF/PBS
8	WF/PBS
9	WF/PBS
10	WF/PBS
11	Bacteria
12	Cull

C: Exposure regime for immunohistochemical analysis; total dose of WF=600µg/mouse

Day	Intervention
1	WF/PBS
2	WF/PBS
3	Bacteria
4	Cull

2.9.2 Welding fume inhalation model

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used in this study. All procedures and protocols were approved by the Animal Care and Use Committee of the National Institute for Occupational Safety and Health. Mice (6

weeks of age) were exposed by whole-body inhalation, in individual steel mesh cages, to aerosols generated during GMA-SS welding for 10 days at a target concentration of 40 mg/m³ for 3 hr/day¹⁵³. The mice were culled and whole lungs removed for mRNA analysis.

The intervention regime for this model is shown in Table 4.

Table 4: programme of intervention for inhalation mouse model.

Day	Intervention
1	Air/WF (40mg/m ³ 3hrs)
2	Air/WF (40mg/m ³ 3hrs)
3	Air/WF (40mg/m ³ 3hrs)
4	Air/WF (40mg/m ³ 3hrs)
5	Air/WF (40mg/m ³ 3hrs)
6	Air/WF (40mg/m ³ 3hrs)
7	Air/WF (40mg/m ³ 3hrs)
8	Air/WF (40mg/m ³ 3hrs)
9	Air/WF (40mg/m ³ 3hrs)
10	Air/WF (40mg/m ³ 3hrs)
11	Cull

RNA was isolated from whole lung homogenates using TRIzol (Invitrogen, USA) and then cleaned according to the manufacturer's instructions using an RNeasy Mini Kit (Qiagen, USA). A 2 µl aliquot of each RNA sample was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). RT-PCR reactions were carried out as follows: Evaluation of gene expression was done using StepOne™ (Applied Biosystems, USA) with pre-designed Assays-on-Demand TaqMan probes and primers (Applied Biosystems). Using 96 well plates, 1µg of total

RNA was reverse transcribed using random hexamers (Applied Biosystems) and Superscript III (Invitrogen, USA). Hypoxanthine-guanine phosphoribosyltransferase was used as an internal reference. Relative gene expression was calculated using the $\Delta\Delta C_t$ method.

2.10 Human model of experimental rhinovirus infection

Endobronchial biopsies were obtained from an experimental RV infection study that recruited human volunteers in London (UK). The biopsies were taken from the lower lobe subcarinae and were snap frozen in liquid nitrogen and stored at -70°C for analysis of PAFR mRNA. Data on bacterial infection, symptoms and antimicrobial peptides have been previously reported⁶⁰. Three groups were studied; i) healthy non-smokers (controls), ii) smokers without airway obstruction, and iii) smokers with moderate COPD (Gold stage II). Bronchoscopy, endobronchial biopsy, bronchoalveolar lavage, and induced sputum were carried out at baseline before human rhinovirus subtype A, serotype 16 (RV-16) inoculation. Endobronchial biopsy was repeated on day 7 after RV inoculation. Informed consent was obtained from all subjects (Research Ethics Committee approval 07/H0712/138, and 11/LO/0400). Rhinovirus infection was assessed by the recruitment team and infection with viruses other than RV was excluded.

2.11 Statistical analysis

Unless otherwise stated, data are expressed as means from at least 3 separate experiments.

Data are analysed using GraphPad Prism version 5.03 (GraphPad Software, La Jolla, California, USA). All cell work was analysed using parametric tests. Single comparisons were carried out using t tests, multiple comparisons were carried out using 1 way ANOVA followed by Bonferroni's multiple comparison test. All animal work was analysed using non-parametric tests. Single comparisons were done by

Mann Whitney U test and multiple comparisons done by Kruskal-Wallis test followed by Dunnett's multiple comparison test. Paired analyses were carried out using paired t tests. In chapter 6, the data set used passed the normality test and hence parametric tests were used, as described above, for analyses. A p value <0.05 was considered significant.

**Chapter 3: Exposure of lower
airway epithelial cells to welding
fumes and susceptibility to
adhesion and invasion by
*Streptococcus pneumoniae***

3.1 Introduction and aims

Epidemiological studies have shown an increased risk of pneumonia and pneumococcal bacteraemia in welders of working age. This increased risk was not seen in retired welders^{4,144}. However, the molecular mechanisms by which exposure to welding fumes (WF) increases susceptibility to pneumococcal pneumonia are unknown.

As reviewed in chapter 1, the first step in the pathogenesis of pneumococcal pneumonia is the adherence of the pneumococcus to airway epithelial cells. This adherence is mediated by adhesins on the pneumococcal surface such as ChoP. ChoP binds PAFR on the surface of human cells and thus co-opts the receptor for pneumococcal entry into the cells⁸. Previous work in our lab showed that exposure to PM₁₀ and CSE causes an increase in PAFR-dependent adhesion to and invasion of lower airway epithelial cells^{86,123}.

It has been shown that exposure of lower airway epithelial cells to welding fume, *in vitro*, caused an increase in ROS produced and a decrease in intracellular glutathione. Further, previous work in our lab showed that the anti-oxidant, N-acetyl cysteine (NAC), attenuated PM₁₀ exposure-induced pneumococcal adhesion and invasion of lower airway epithelial cells⁸⁶. This suggests that apart from PAFR, oxidative stress mechanisms may also be involved in particulate matter induced pneumococcal adhesion and invasion of these cells.

Hence, the questions I sought to address were:

- Are airway epithelial cells exposed to welding fume more susceptible to pneumococcal invasion and adhesion?
- Is increased susceptibility PAFR-dependent?
- Does oxidative stress play a role in increased susceptibility?

3.2 Results

3.2.1 Exposure of A549, BEAS-2B and RPMI2650 cells to welding fume and pneumococcal adhesion and invasion

In order to assess whether exposure to welding fumes increases susceptibility of lower airway cells to adhesion and invasion by pneumococci A549 and BEAS-2B cells were exposed to different doses (50-400µg/ml) of mild steel (MS) and stainless steel (SS) welding fumes (WF). The fumes were then washed off and the cells infected with *S. pneumoniae* strain D39 at an MOI of 100. Using data from these experiments optimal doses of welding fume for each cell line were chosen for future work. The number of adherent and invaded bacteria for each dose of welding fume was compared to the control using an unpaired t test in order to determine the lowest dose at which statistical significance was observed.

Exposure of A549 and BEAS-2B cells to welding fume increased pneumococcal adhesion and invasion of these cells in a dose dependent manner (figure 1). In A549 cells exposed to mild steel WF the lowest dose at which significance was seen, compared to the control using an unpaired t test, was 300µg/ml (*p<0.05, figure 1A). For A549 cells exposed to stainless steel WF this dose was 200µg/ml (*p<0.05, figure 1C). In A549 dose response experiments with mild steel WF (n=4) some changes in cell morphology, i.e loss of adherence of the cells, were seen in cells exposed to mild steel WF at a dose of 300µg/ml (n=1). Hence, 275µg/ml was chosen as optimal dose for additional experiments in A549 cells. In BEAS-2B cells exposed to mild steel WF the lowest dose at which a significant increase in bacterial adhesion and invasion was seen, compared to the control using an unpaired t test, was 200µg/ml (**p<0.01, figure 1B). In BEAS-2B cells exposed to stainless steel WF this dose was 50µg/ml (*p>0.05, figure 1D). Hence, a dose of 200µg/ml dose was chosen for further experiments.

Maximum pneumococcal adhesion and invasion seen was higher in A549s (approximately 5500 CFU/ml) compared to BEAS-2Bs (2000 CFU/ml).

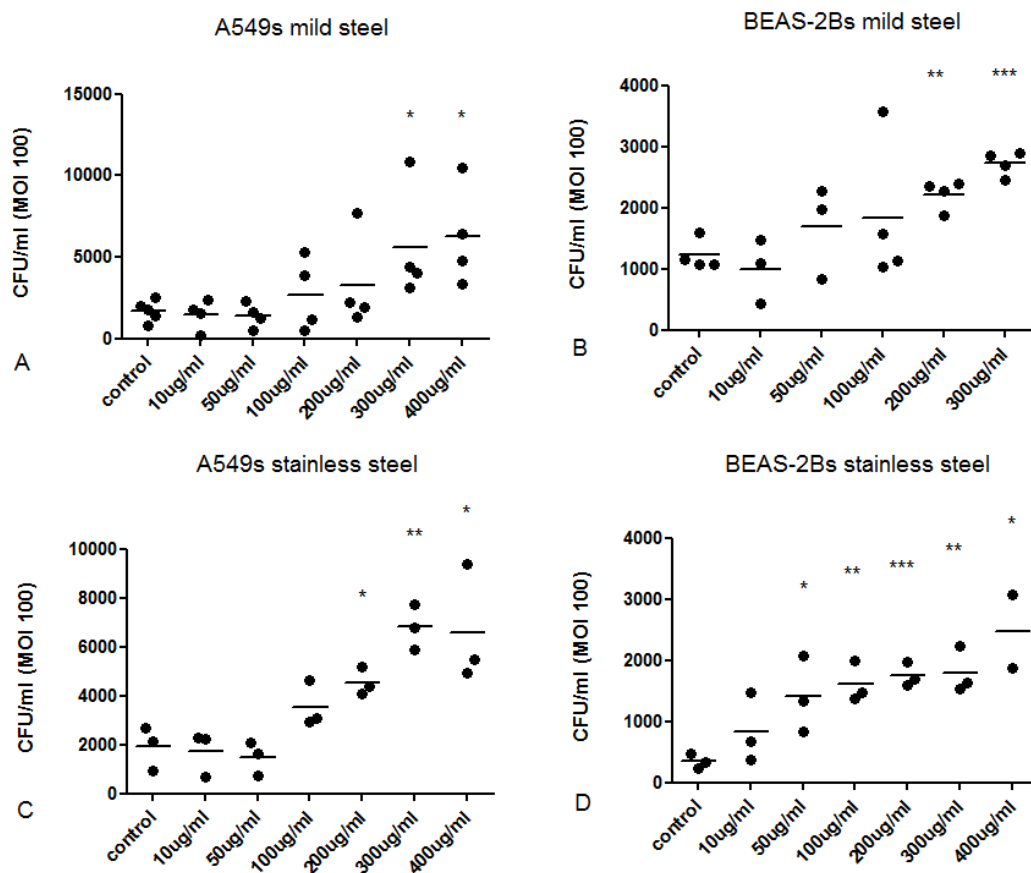


Figure 1: Pneumococcal adhesion and invasion of (A) A549 cells exposed to mild steel WF (B) BEAS-2B cells exposed to mild steel WF (C) A549 cells exposed to stainless steel WF, and (D) BEAS-2B cells exposed to stainless steel WF. Data are from at least 3 separate experiments and are expressed as averages from at least 4 wells/experiment. Data are compared to the control using unpaired t tests and 1 way ANOVA followed by Dunnett's multiple comparison test.

Nasal carriage of *S. pneumoniae* is high in the general population and translocation of pneumococci across the nasal epithelial barrier may lead to bacterial invasion of the blood stream. Hence, it was considered important to assess the effect of

exposure to WF on pneumococcal adhesion to and invasion of nasal epithelial cells and thus susceptibility to pneumococcal disease.

The cells were exposed to a range of doses (10-300µg/ml) of mild steel welding fume. The WF was then washed off and the cells infected with *S. pneumoniae*. The cells were then lysed and adherent and invaded pneumococci analysed.

Using an unpaired t test it was found that the lowest dose at which a significant increase in adhesion and invasion was observed compared to the control was 200µg/ml (*p<0.05). However, since no changes in cell morphology were seen at 300µg/ml, this dose was used in future experiments (figure 9). The maximum stimulation of pneumococcal adhesion and invasion seen in these experiments was approximately 3500 CFU/ml which is higher than that seen in BEAS-2B (2000 CFU/ml) cells but lower than that seen in A549 cells (5500 CFU/ml).

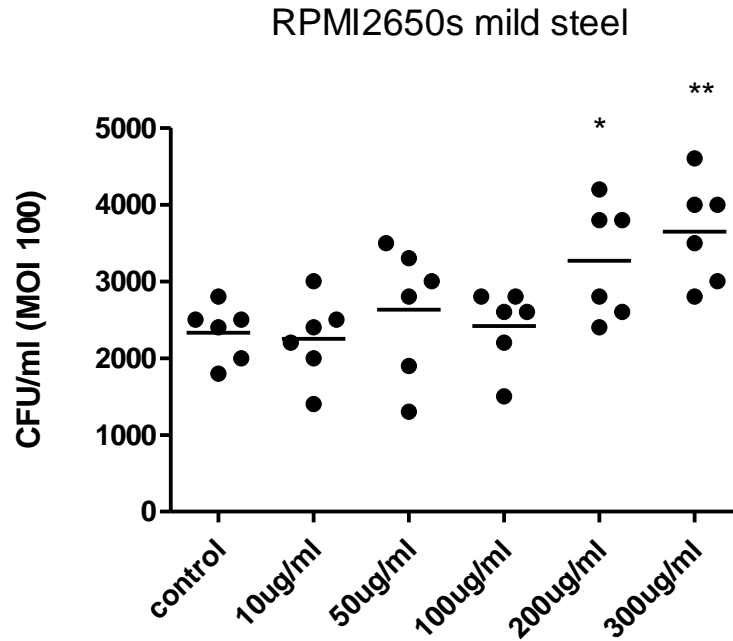
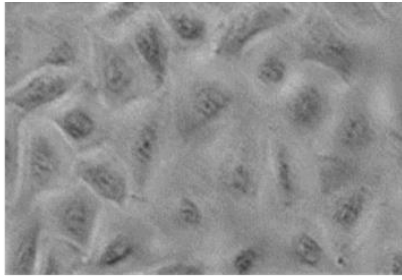
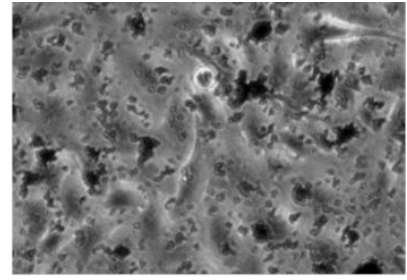


Figure 2: Pneumococcal adhesion and invasion of nasal epithelial cells after exposure to a range (50-300 μ g/ml) of doses of mild steel welding fume. Pneumococcal adhesion and invasion to these cells increased in a dose-dependent manner after exposure to the WF. The lowest dose at which a significant increase in pneumococcal adhesion and invasion was seen compared to the control was 200 μ g/ml. Data are from two separate experiments. Each data point represents a single sample. Data were compared using unpaired t tests and a 1 way ANOVA followed by Dunnett's multiple comparison test.

The welding fume dust was in suspension in PBS and was diluted down to a concentration of 275 μ g/ml in complete media as described in the methods chapter of this thesis. Each particle of WF is less than 10 μ m in diameter. The WF coats the cells (figure 3) with some larger clumps being formed. No morphological changes were seen after the cells were exposed to WF at this dose for 2 hours.



A



B

Figure 3: Phase contrast microscopy images of A549 cells (A) grown in complete media, (B) exposed to 275ug/ml mild steel welding fume. The welding fume coats the cell monolayer with some larger clumps of particulate matter being formed.

The effect of exposure to mild steel welding fume on bacterial growth was also assessed by growing the bacteria in the presence and absence of welding fume. CFU/ml was assessed after 3hours, using quantitative bacterial culture. A decrease in bacterial growth was observed in pneumococcal stocks grown in welding fume for 3h, compared to the control (figure 4). However, this decrease was not significant. The dose of WF used in this experiment is the highest dose used throughout the studies presented in this thesis i.e 300µg/ml.

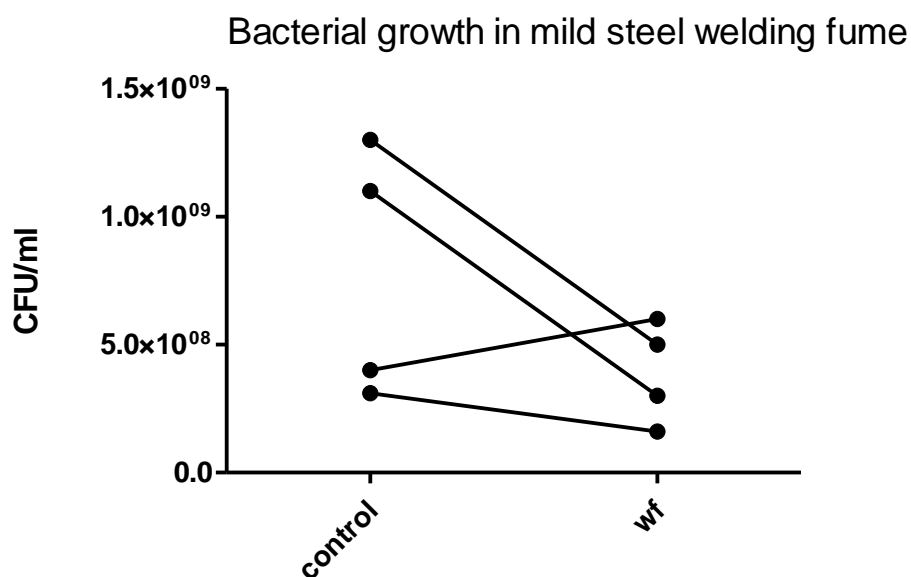


Figure 4: Effect of 3h exposure to WF on bacterial growth. CFU/ml observed after pneumococcal growth in WF from stocks stored at -80°C was lower compared to the control. However, the difference in CFU/ml was not significant. Data are from 4 separate experiments and are compared using a paired t test.

3.2.2 WF and viability of cells

Cell viability over the range of WF doses used in this study was measured using lactate dehydrogenase (LDH) leakage into the media (figure 5). LDH is a marker of tissue damage. This was done to check whether bacterial adhesion and invasion of the cells after exposure to WF could be attributed to loss of membrane integrity of these cells.

No significant effect on cell viability was seen. Distilled water was used as a positive control for cell viability because it anti-oxidantlyses cells by osmotic shock.

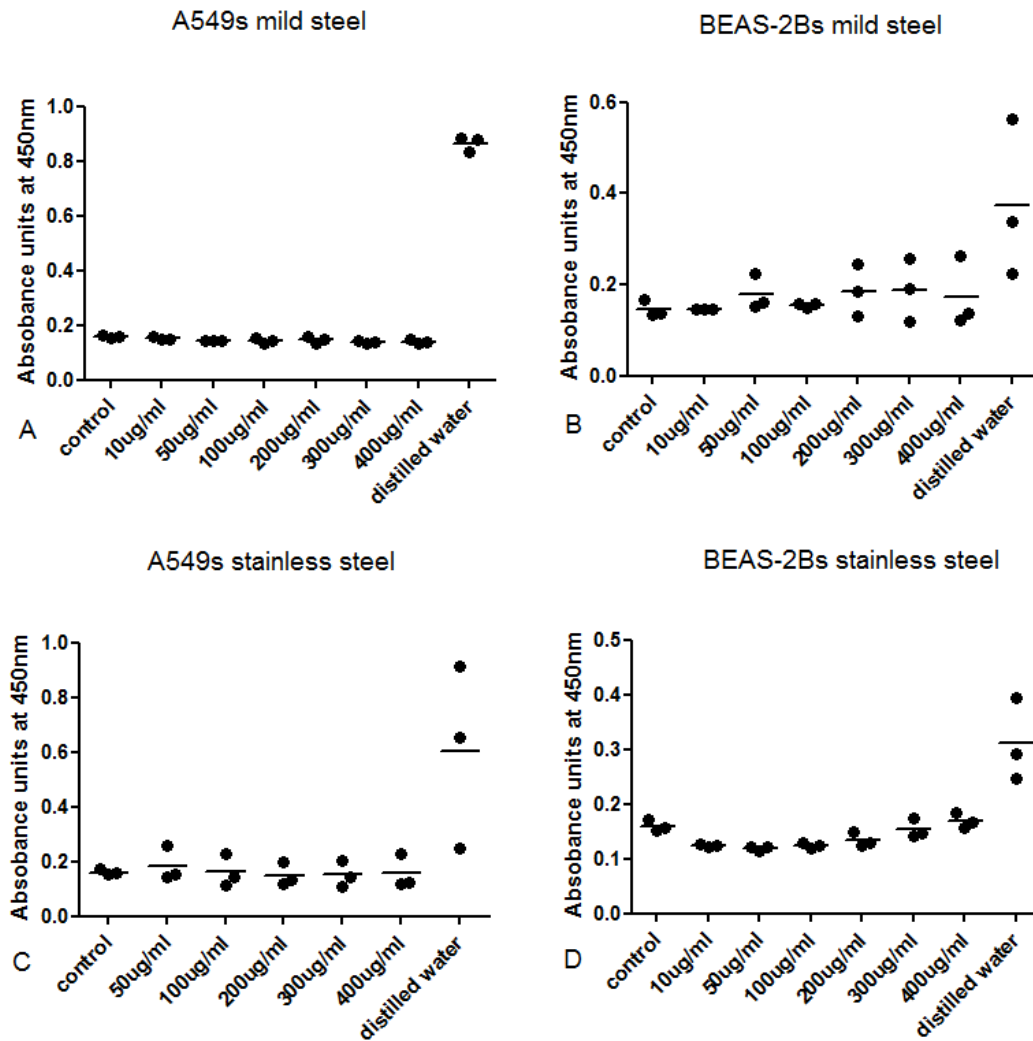


Figure 5: Cell viability effects after exposure to welding fume. Mild steel welding fume exposure in (A) A549s (B) BEAS-2Bs, and stainless steel WF exposure in (C) A549s (D) BEAS-2Bs. Data are from single experiments. In each experiment data from 3 wells are expressed.

3.2.3 Role of oxidative stress in mediating WF-simulated pneumococcal adhesion

The anti-oxidant, NAC, was used to assess the role of oxidative stress in WF stimulated pneumococcal adhesion and invasion of lower airway epithelial cells. Exposure to WF causes oxidative stress in lower airway epithelial cells and hence an increase in oxidative stress may contribute to increased susceptibility of these

cells to pneumococcal adhesion and invasion. NAC replenishes glutathione stores. Glutathione is a biological antioxidant. The cells were exposed to either NAC alone, WF alone or NAC + WF for 2 hours before infection with *S. pneumoniae*.

WF-stimulated pneumococcal adhesion was attenuated by treatment with NAC during exposure of the cells to welding fume (figure 6). A low level of basal adhesion and invasion was seen in the control cells and was also observed in cells exposed to NAC. A significant increase in pneumococcal adhesion and invasion was seen in cells exposed to WF alone compared to the control in all experiments. A significant decrease in adhesion and invasion was seen in cells exposed to NAC + WF compared to cells exposed to WF alone in all experiments.

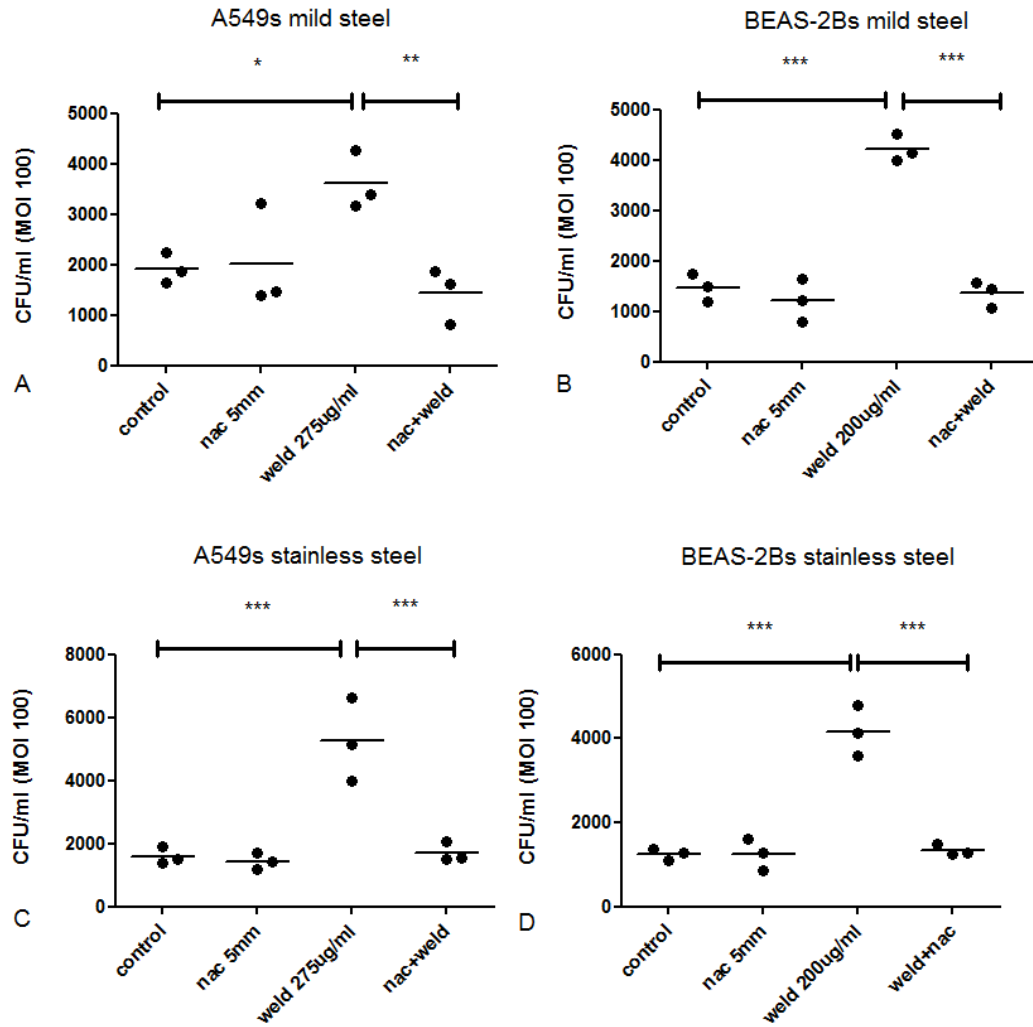


Figure 6: Treatment of cells with NAC during exposure to welding fume causes an attenuation of welding fume-stimulated pneumococcal adhesion to and invasion of lower airway epithelial cells. NAC attenuated mild steel WF-stimulated adhesion to cells in (A) A549s (** $p < 0.01$) (B) BEAS-2Bs (** $p < 0.001$), and stainless steel exposure-stimulated pneumococcal adhesion in (C) A549s (** $p < 0.001$) (D) BEAS-2B cells (** $p < 0.01$). In all experiments, a significant increase in adhesion and invasion was seen after exposure to WF compared to control. Data are from 3 separate experiments and are expressed as means from >4 wells from each experiment. Data are compared using 1 way ANOVA followed by Bonferroni's multiple comparison test.

3.2.4 Role of PAFR in mediating WF-simulated pneumococcal adhesion

A specific PAFR blocker, CV-3988, was used to assess the role of PAFR in WF-stimulated pneumococcal adhesion and invasion of lower airway epithelial cells. The hypothesis was that since CV-3988 binds PAFR it would block the interaction between PAFR and ChoP on the pneumococcal surface and thus prevent the internalisation of pneumococci via PAFR. The cells were exposed to either 20µM CV-3988 alone, WF alone or CV-3988 + WF for 2 hours. The blocker was washed off before pneumococcal infection.

WF-stimulated pneumococcal adhesion was attenuated by treatment with CV-3988. A low level of basal adhesion and invasion seen in the control cells was also observed in cells treated with CV-3988. In all experiments a significant increase in pneumococcal adhesion and invasion was seen after WF stimulation, compared to the control. A significant decrease in pneumococcal adhesion and invasion was seen in cells exposed to WF + CV-3988 compared to cells exposed to WF alone (figure 7).

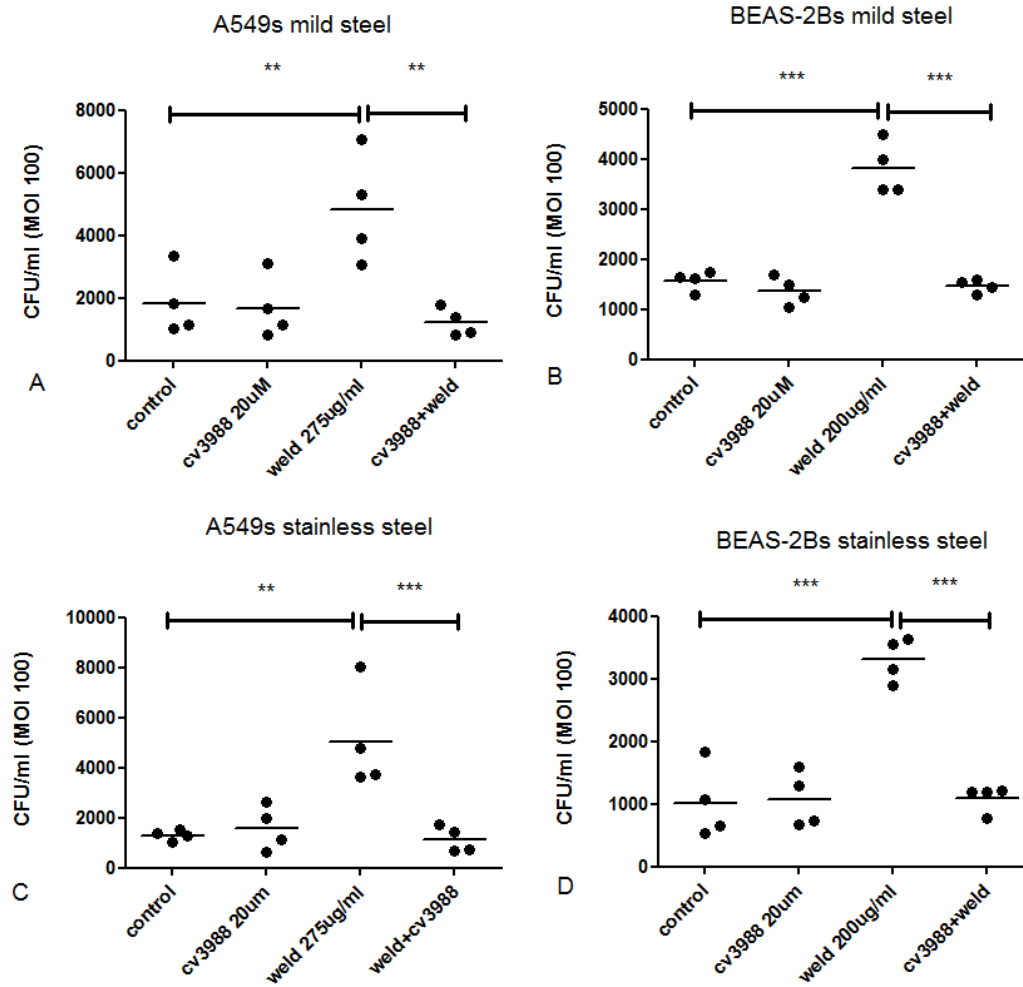


Figure 7: Treatment with CV-3988 during exposure of cells to weld fume causes an attenuation of weld fume-stimulated pneumococcal adhesion to the cells. CV-3988 attenuated mild steel WF exposure-stimulated pneumococcal adhesion in (A) A549s (** $p < 0.01$) (B) BEAS-2B cells (** $p < 0.001$), and stainless steel exposure-stimulated pneumococcal adhesion in (C) A549 cells (** $p < 0.001$) (D) BEAS-2Bs (** $p < 0.001$). In all experiments, a significant increase in adhesion and invasion was seen after exposure to WF compared to control. Data are from 3 separate experiments and are expressed as means from >4 wells from each experiment. Data are compared using 1 way ANOVA followed by Bonferroni's multiple comparison test.

It was also considered important to test whether PAFR has a role in WF-induced pneumococcal adhesion and invasion to nasal epithelial cells. In order to do this the cells were exposed to CV-3988, a PAFR blocker, concurrent to WF exposure in the adhesion assay outlined above and in the methods chapter of this thesis.

Exposure of nasal epithelial cells to CV-3988 concurrent to WF-exposure attenuated WF-induced pneumococcal adhesion and invasion of the cells (figure 8, $^{**}p<0.01$). As with the A549 and BEAS-2B cells, a low level of basal adhesion and invasion was seen in the control cells, which was not attenuated by treatment with CV-3988 alone.

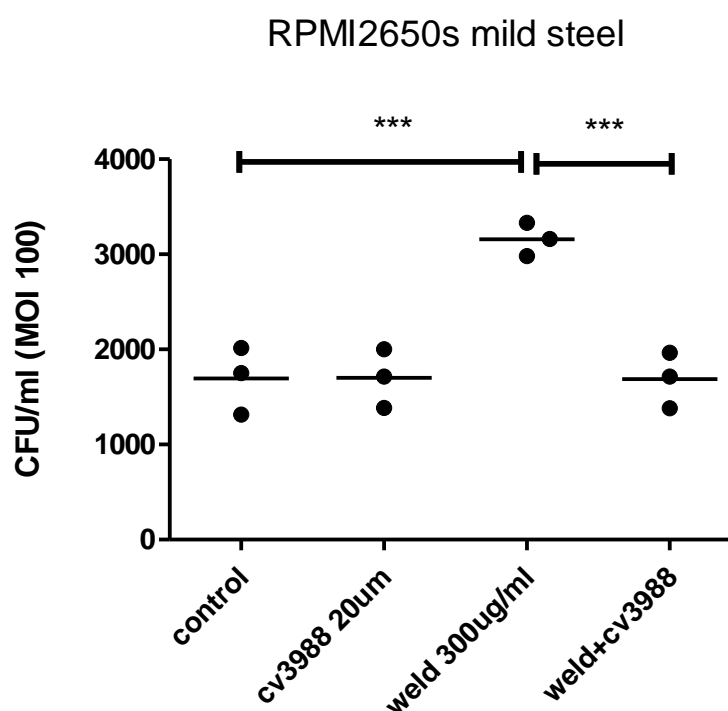


Figure 8: Concurrent treatment of cells with WF and CV-3988 attenuated WF-induced pneumococcal adhesion and invasion to nasal epithelial cells ($n=3$, $^{***}p<0.001$). Each data point is the mean of at least 4 samples and represents a single experiment. The data were compared using 1 way ANOVA followed by Bonferroni's multiple comparison test.

3.2.5 WF and PAFR mRNA expression

Since previous data showed a role of PAFR in WF-stimulated pneumococcal adhesion and invasion, PAFR expression levels, after a 2hour WF exposure, were studied at the mRNA level. It was hypothesised that PAFR mRNA is upregulated after WF exposure which increases susceptibility to pneumococcal infection.

PAFR mRNA expression was measured after exposure to WF. For each sample PAFR mRNA expression was measured relative to the housekeeping gene, GAPDH. Each data point represents a single experiment and is the average of at least 2 samples. Expression levels were measured using TaqMan primers and probes and the $\Delta\Delta C_t$ method.

An increase in PAFR expression was only seen in A549 cells after exposure to mild steel WF. No change in mRNA levels was seen in A549 cells after exposure to stainless steel WF or in BEAS-2B cells after exposure to either type of WF (figure 9).

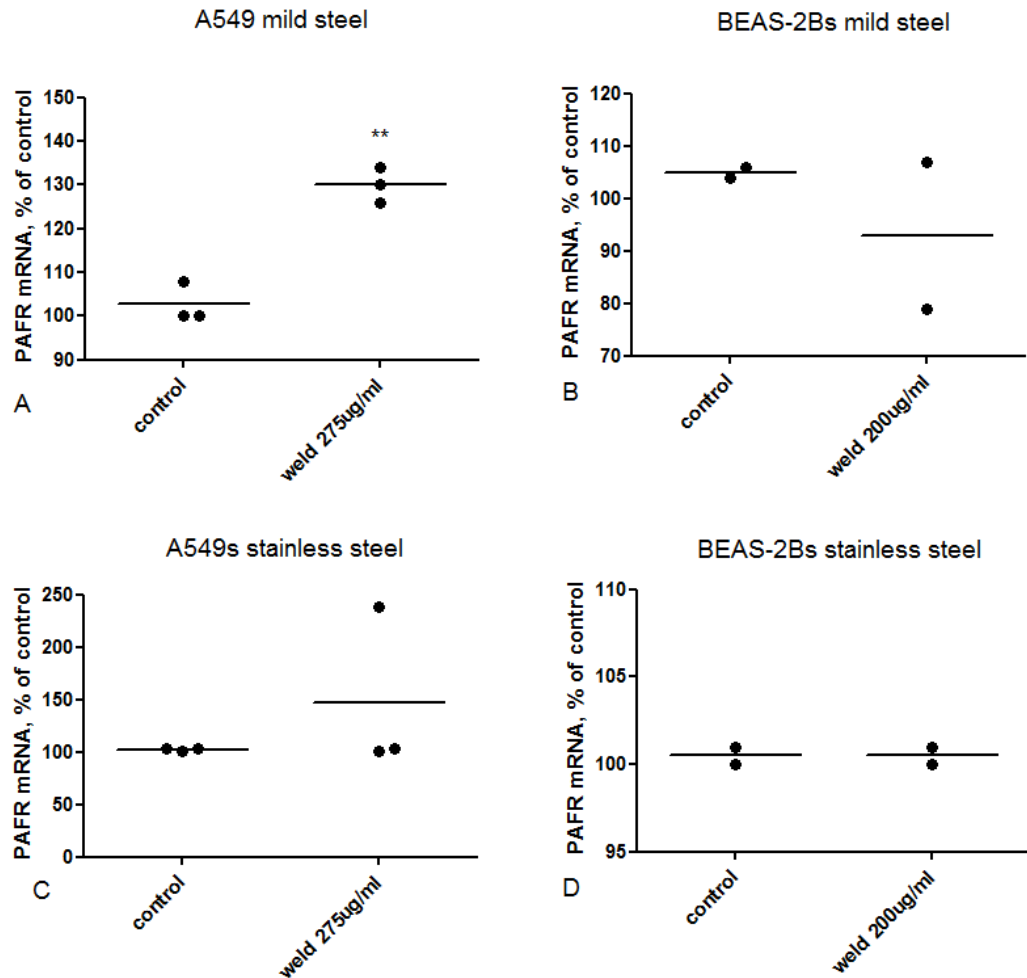


Figure 9: mRNA expression of PAFR after exposure to WF. In (A) an increase was seen in A549 cells after exposure to mild steel WF (** $p < 0.01$). No significant change in expression was seen in (B) A549 cells after exposure to stainless steel WF or in BEAS-2B cells after exposure to (C) mild steel WF or (D) stainless steel WF. Data are from at least 2 separate experiments and are expressed as means from at least 2 samples per experiment. Data are compared using unpaired t tests.

3.2.6 WF and surface protein expression of PAFR

Since examining PAFR expression at the mRNA level proved inconclusive and PAFR is a surface protein it was important to assess PAFR protein expression on the surface of cells after exposure to WF. Since the role of PAFR in WF-induced pneumococcal adhesion and invasion was directly assessed using the PAFR

blocker, CV-3988, for both types of WF, PAFR protein levels were only assessed in both cell types using mild steel WF.

Surface protein expression of PAFR was measured using immunofluorescence microscopy. The cells were exposed to WF for 2 hours. The WF was washed off and the cells fixed. A mouse anti-human PAFR primary antibody was used followed by an Alexa-fluor 488 secondary antibody. DAPI was used to detect nuclear DNA. To check for non-specific staining a mouse isotype control antibody was used instead of the primary antibody (figure 10). The green areas seen in the figure are most likely clumps of secondary antibody rather than non-specific binding because they are not in the immediate vicinity of cells. This experiment showed that interactions between the anti-human PAFR antibody and the secondary antibody detected by immunofluorescence microscopy were specific.

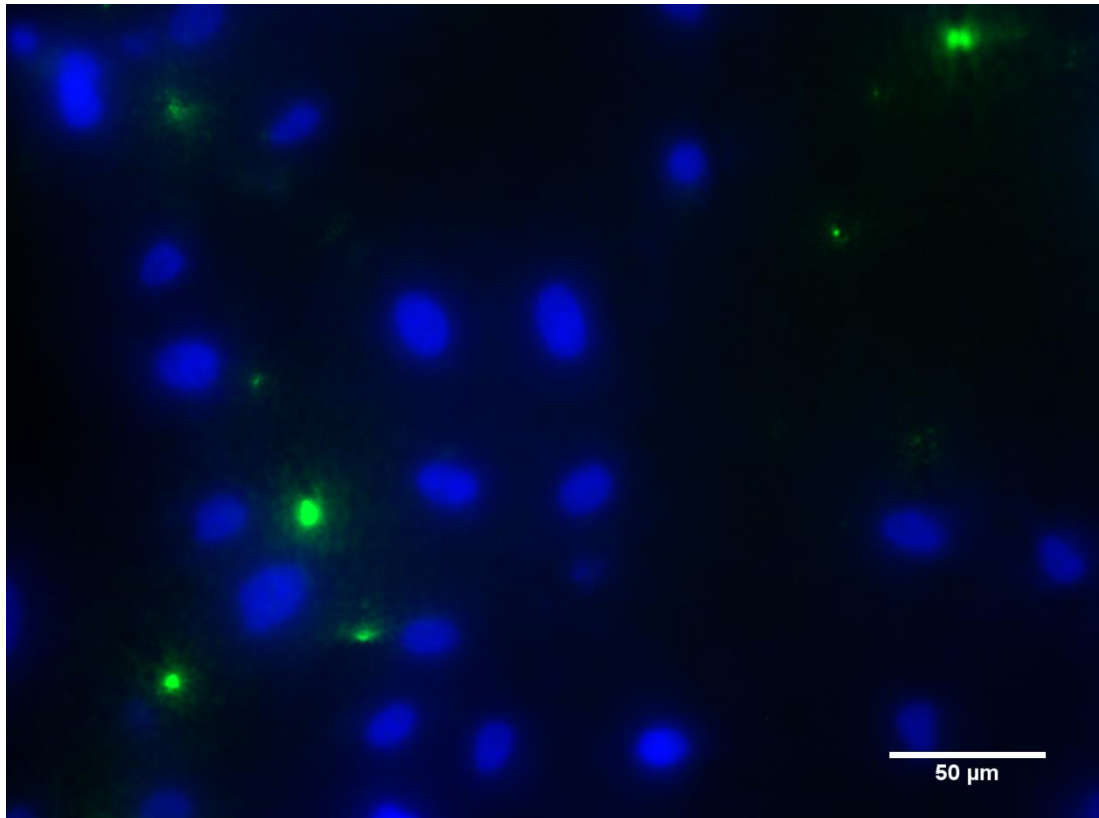


Figure 10: Non-specific binding of the secondary antibody to A549 cells. An isotype control was used instead of the primary anti-human PAFR antibody, followed by the secondary antibody to check for non-specific binding of the secondary antibody to the cells. The nuclei of the cells are stained with DAPI (blue). No secondary antibody staining (green) was seen associated with cells which suggests that the staining seen is due to clumping of the secondary antibody rather than due to non-specific interactions between primary (anti-PAFR) and secondary antibodies.

An example of PAFR surface expression in A549 cells in complete media or exposed to WF can be seen in figure 11. The images were analysed in a blinded manner. A fluorescence intensity threshold was set to discount background fluorescence. The area of fluorescence was then measured for each image. 1-3 images/condition were analysed in each experiment. In the images presented DAPI and PAFR staining was merged to show localisation of PAFR. However, for the

analyses, PAFR staining was measured separately to prevent DAPI staining affecting the fluorescence intensity readings.

A significant increase in PAFR surface expression was seen in A549 cells after exposure to mild steel WF (* $p < 0.05$, figure 11A). A trend towards higher expression levels of PAFR surface protein was seen in BEAS-2B cells after exposure to mild steel WF ($p = 0.06$, figure 11B). An example of the staining observed can also be seen in figure 11C and D.

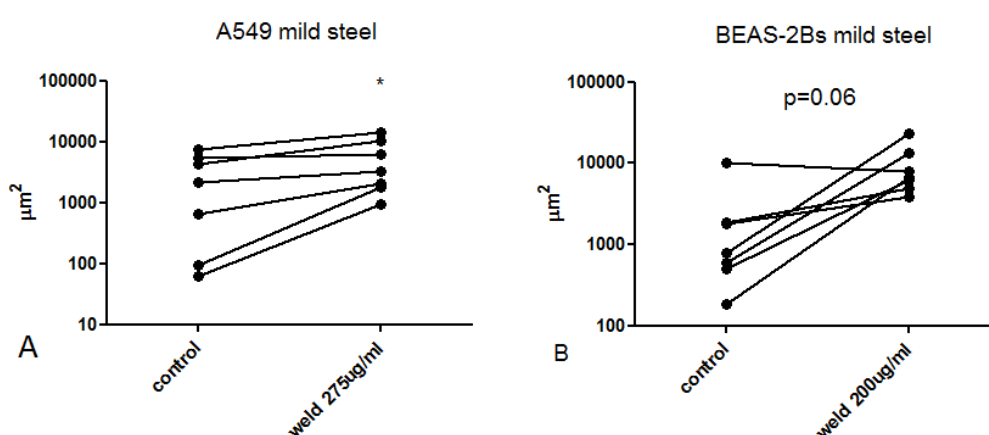


Figure 11A;B: PAFR surface expression in lower airway epithelial cells after exposure to mild steel WF. An increase in expression was seen after exposure to welding fume, compared to the control in (A) A549 cells (* $p < 0.05$). A trend towards higher expression was seen in (B) BEAS-2B cells ($p = 0.06$). Data are from 7 separate experiments. Data from 1-3 samples per condition were averaged in each experiment and each data point represents one experiment. The data were compared using paired t tests. The images were analysed using ImageJ software and the PAFR stained area of each image was calculated.

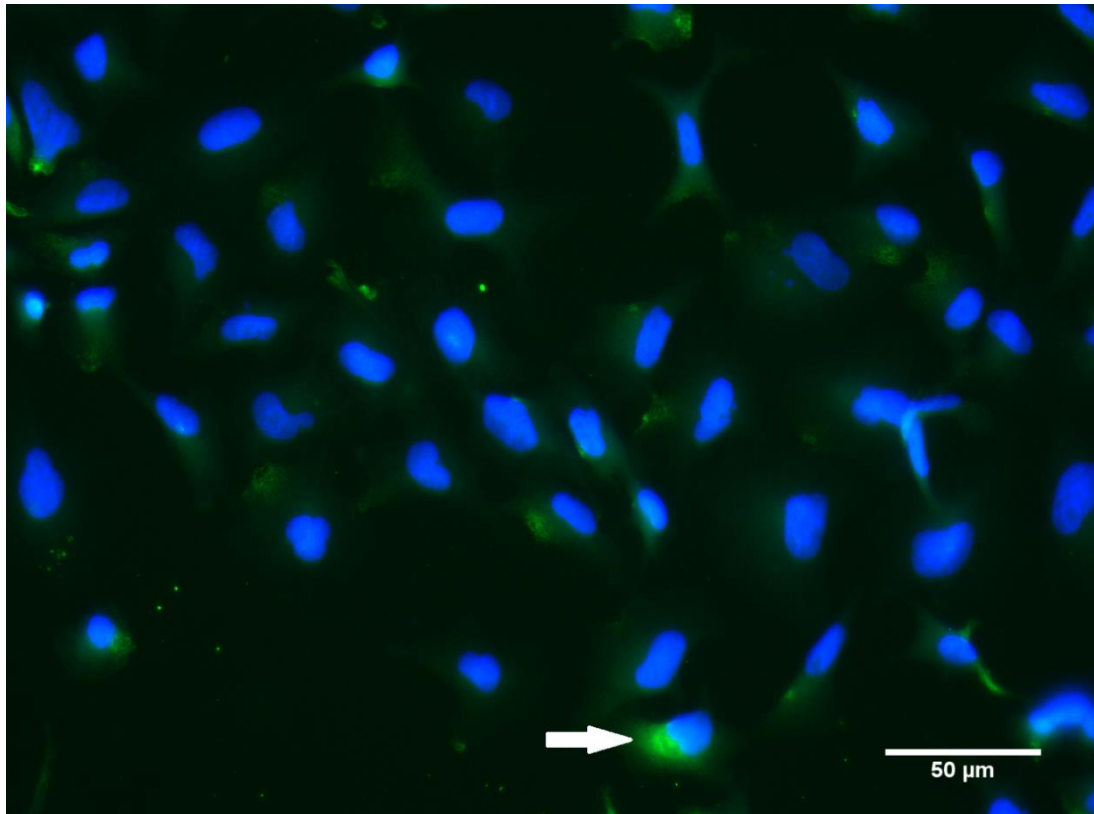


Figure 11C: An example of PAFR staining can be seen in control A549 cells. The white arrow denotes an example of an area of PAFR staining included in the analysis.

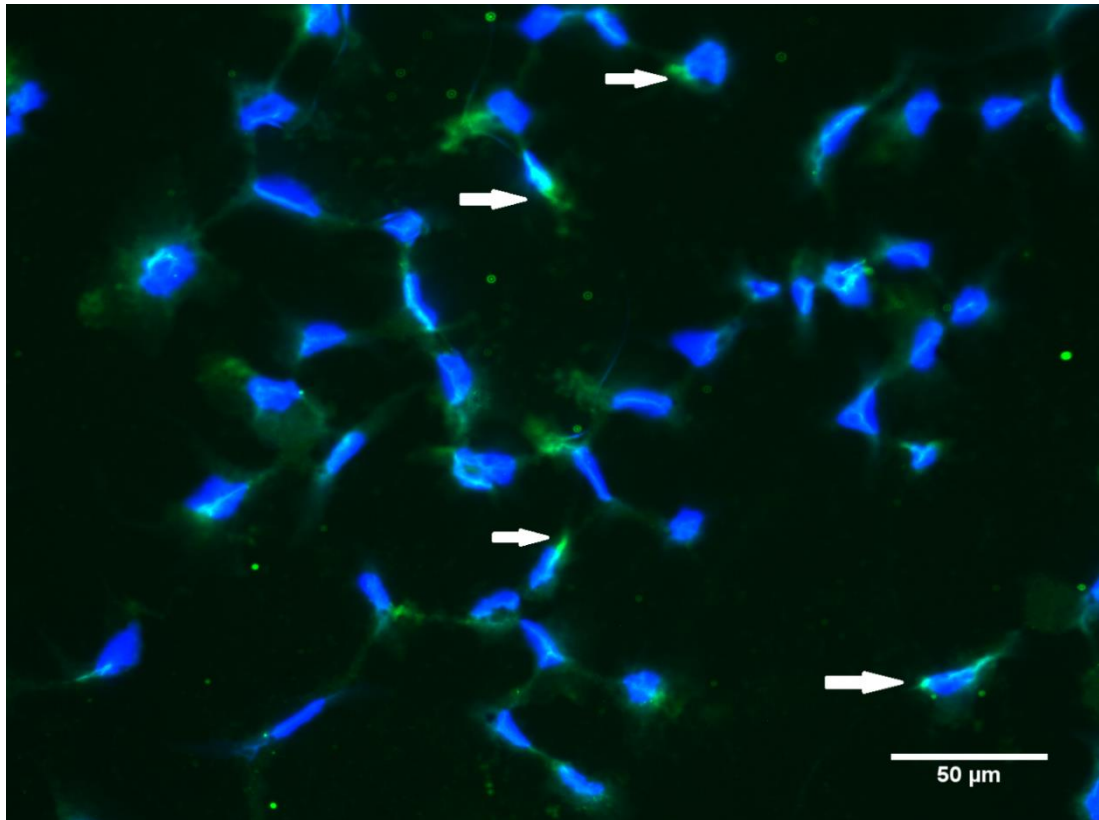


Figure 11D: An example of PAFR staining can be seen in WF exposed A549 cells. The white arrows denote examples of areas of PAFR staining included in the analysis.

3.3 Conclusions

The data show that in A549 and BEAS-2B cells exposure to welding fume increases susceptibility to adhesion and invasion by *S. pneumoniae* in a dose-dependent manner. Similar dose response curves were observed for both mild steel and stainless steel welding fume. An optimal dose of 275μg/ml for A549 cells and 200μg/ml for BEAS-2B cells was chosen for further studies, based on the dose response data. In this study, cell viability was also measured, using the lactate dehydrogenase release assay, over the range of doses used. No effect on cell viability after exposure to either type of WF was seen in either cell line. This is contrary to a previous report that exposure to stainless steel WF caused a decrease in cell viability at doses above 63μg/ml¹⁴¹. Cell viability in the study was measured in

A549 cells using a similar assay to that used for this thesis¹⁴¹. This observation highlights the importance of evaluating the effects of the different components of the WF on airway epithelial cell biology.

CV-3988, which binds PAFR and thus blocks its interaction with ChoP on the surface of pneumococci, was used to show that the increased susceptibility to pneumococcal invasion and adhesion after exposure to WF is PAFR-dependent. This observation is consistent with previous studies conducted in our lab which showed that PAFR is a key receptor involved in increased susceptibility to *S. pneumoniae* adhesion to lower airway epithelial cells after exposure to PM₁₀ and CSE^{86,123}. However, it was observed that a low level of basal adhesion and invasion occurred in spite of treatment with CV-3988. This basal adhesion and invasion was also seen in the control cells^{8,43}. This suggests that there may be some yet unknown PAFR-independent mechanisms involved in this process. Further work is required to provide clues about what these mechanisms may be.

Exposure to WF has been shown to cause oxidative stress in lower airway epithelial cells. Further, previous work in our lab showed that an attenuation of oxidative stress, caused by exposure to PM₁₀, by NAC caused an attenuation of *S. pneumoniae* invasion of lower airway epithelial cells⁸⁶. Additionally, it has been shown that NAC attenuated increased ROS production by bronchial epithelial cells after exposure to PM⁶⁸ and injury caused by cigarette smoke in murine alveolar epithelial cells¹⁰³. Hence, it was hypothesised that an anti-oxidant such as N-acetyl cysteine may attenuate WF-stimulated pneumococcal adhesion and invasion of lower airway epithelial cells. Indeed treatment of the cells with NAC concurrent to WF exposure attenuated WF-stimulated pneumococcal infection. Again, as with the use of CV-3988, the basal adhesion and invasion of the cells was not affected by treatment with NAC.

In A549 cells it was shown that exposure to mild steel welding fume caused an increase in mRNA expression of PAFR. These data are consistent with the hypothesis that exposure to the welding fume causes an upregulation of PAFR expression which in turn leads increased pneumococcal invasion of the cells. However, this upregulation of PAFR mRNA was not seen in A549 cells exposed to stainless steel welding fume and in BEAS-2B cells exposed to either mild steel or stainless steel welding fume. Hence, taken together the PAFR mRNA data were inconclusive. PAFR is a surface receptor. PAFR signalling involves rapid internalisation after ligand binding and recycling of the receptor. The process of transcription is relatively much slower and the mRNA may be quickly translated because it is not very stable in the cytoplasm. Further work is required to better understand the kinetics of this process. However, for the purpose of this investigation it was considered more relevant to study surface protein expression instead.

Using fluorescent microscopy it was shown that exposure to WF significantly increases PAFR surface expression in lower airway epithelial cells. Hence, there is evidence to support the hypothesis that exposure to WF increases PAFR surface expression and thus increases susceptibility to pneumococcal infection in these cells. As mentioned earlier, PAFR is rapidly internalised after ligand binding and is recycled. The kinetics of this process are poorly understood. Further, very little is known about the downstream signalling processes.

S. pneumoniae is carried asymptotically in the nasopharynx by a large percentage of the population. Translocation of pneumococci across the nasal mucosal barrier may lead to pneumococcal bacteraemia. Additionally, PAFR expression has previously been observed in nasal epithelial cells¹¹. In light of the epidemiological evidence that welders are more susceptible to pneumococcal bacteraemia it was considered of importance to assess the effect of exposure of

nasal epithelial cells to WF on pneumococcal adhesion and invasion of these cells. The data presented earlier in this chapter suggest that exposure to mild steel welding fume increases susceptibility of nasal epithelial cells to pneumococcal infection. As the nasal epithelial cells responded to WF exposure in a similar manner to the A549 and BEAS-2B cells, only one type of WF was tested in these preliminary experiments. The data also suggest a role of PAFR in the increased susceptibility. This suggests that PAFR expression in nasal cells exposed to WF may be a good biomarker for susceptibility to pneumococcal infection in the lower airway. To expand on this preliminary work in nasal epithelial cells, it would be of interest to examine PAFR expression in the nasal mucosa of welders who have recently been involved in welding activity.

Pneumococcal adhesion to airway epithelial cells is the first step of pneumonia pathogenesis. Occupational exposure to welding fume has been shown to increase risk of pneumococcal pneumonia. In this study it has been shown that exposure to WF increases PAFR expression on the surface of lower airway epithelial cells and that blocking PAFR attenuates pneumococcal adhesion and invasion in both upper and lower airway epithelial cells. Hence, PAFR may be a putative anti-infective target in welders. Further work is required to better understand the kinetics of PAFR expression and signalling and to elucidate the mechanisms by which oxidative stress plays a role in increased susceptibility of lower airway epithelial cells to pneumococcal infection. However, a major limitation of this study is that it was carried out using immortalised cell lines. Modification of primary cells or tissue by immortalisation causes changes in the genetic and phenotypic characteristics of the cells¹⁵⁴. Additionally, the study looks at the effect of WF on each type of cell in isolation and does not model any complex interactions between cell types or between the cells used and other components of the respiratory or immune system, such as macrophages. Hence, the data presented in this chapter may not be an

accurate representation of what is occurring *in vivo*. Primary bronchial epithelial cells are currently commercially available but could not be used in this study due to financial restraints. It is of interest to validate the findings presented in this chapter in these primary cells.

Chapter 4: Exposure to welding fume and susceptibility to pneumococcal pneumonia in mice

4.1 Introduction and aims

Occupational exposure to welding fume has been shown to increase susceptibility to pneumococcal pneumonia. However, biological plausibility has not been established. Further, little is known about the mechanisms that confer this increased susceptibility.

It has been shown that rats chronically exposed to welding fumes have increased DNA damage in the lungs¹⁴⁰ and increased numbers of inflammatory cells such as neutrophils and macrophages^{136,139}. Further, it has been shown that rats exposed to welding fumes and infected with *L. monocytogenes* have a higher bacterial burden^{145,153}.

Data presented in chapter 3 showed that increased pneumococcal adhesion and invasion of welding fume exposed lower airway epithelial cells is PAFR-dependent and that welding fume exposure increased PAFR surface protein expression in these cells.

Hence, this study sought to determine whether the results seen *in vitro*, outlined in chapter 3, translate to a biological effect *in vivo*. Thus the study had the following aims:

- To establish biological plausibility of increased susceptibility to pneumococcal infection after welding fume exposure in a mouse model
- To assess PAFR expression and localisation in the lungs of welding fume exposed mice

4.2 Results

4.2.1 Acute exposure to WF by intranasal inoculation route, *S. pneumoniae* strain: D39

Mice (n=6/group) were intranasally inoculated with either PBS; mild steel WF; PBS + *S. pneumoniae* or WF + *S. pneumoniae*, under anaesthesia. 600µg of WF and 5×10^6 *S. pneumoniae* were used. The total amount of inoculum/mouse was 50µl. The amount of pneumococci used was measured using quantitative bacterial culture. The mice were culled 24 hours later for analysis of CFU counts in the blood, BALF and one lung. The second lung was used for PAFR mRNA analysis. A tabular representation of the timing and type of intervention performed is shown below.

Day	Intervention
1	Intranasal inoculation of PBS+D39/WF+D39 under anaesthesia
2	Cull

A significant increase in both lung (**p<0.01, figure 1B) and BALF CFU counts was observed after exposure to welding fume compared to the control (*p<0.05, figure 1A). Further, a significantly higher proportion of the leucocyte population found in the lungs of mice that received WF alone was neutrophils compared to the PBS control (*p<0.05, figure 1C). Similarly, a significantly higher proportion of the leucocyte population of the lungs of mice that received WF+bacteria was neutrophils compared to those that received PBS+bacteria (*p<0.05, figure 1C). No pneumococci were observed in the blood.

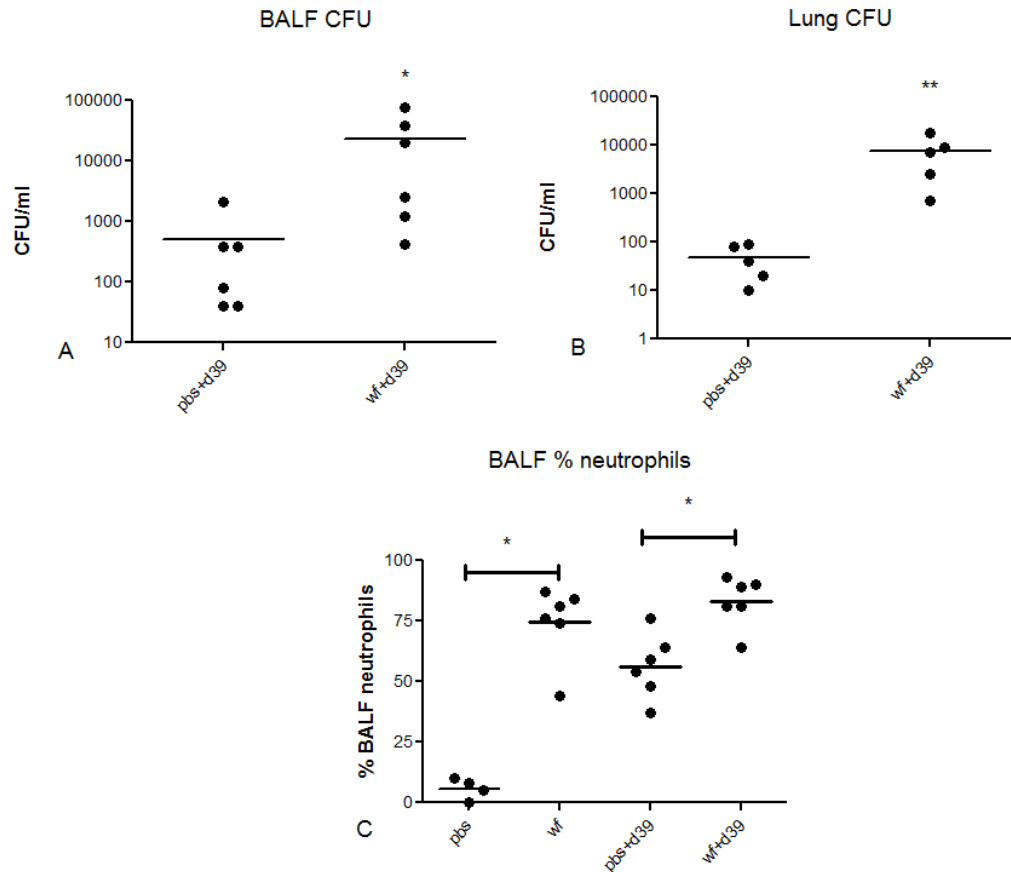


Figure 1: Exposure to mild steel welding fume increases susceptibility to pneumococcal pneumonia in mice (n=6/group). Increased CFU counts were observed in (A) BALF and (B) lung. (C) A higher percentage of neutrophils was seen also in mice exposed to welding fumes and infected with pneumococci compared to those only infected with pneumococci. CFU data are compared using Mann-Whitney U tests, neutrophil data are compared by Kruskal-Wallis test followed by Dunn's multiple comparison test.

In the same experiment PAFR mRNA expression levels were assessed using TaqMan primers and probes. Gene expression was measured relative to the housekeeping gene and analysed using the $\Delta\Delta C_t$ method. RT-PCR reactions were carried out in triplicate.

A significant increase in PAFR expression was seen after exposure to WF alone (10 fold, * $p<0.05$) and to WF+bacteria (approximately 7 fold, ** $p<0.01$) compared to the PBS control (figure 2).

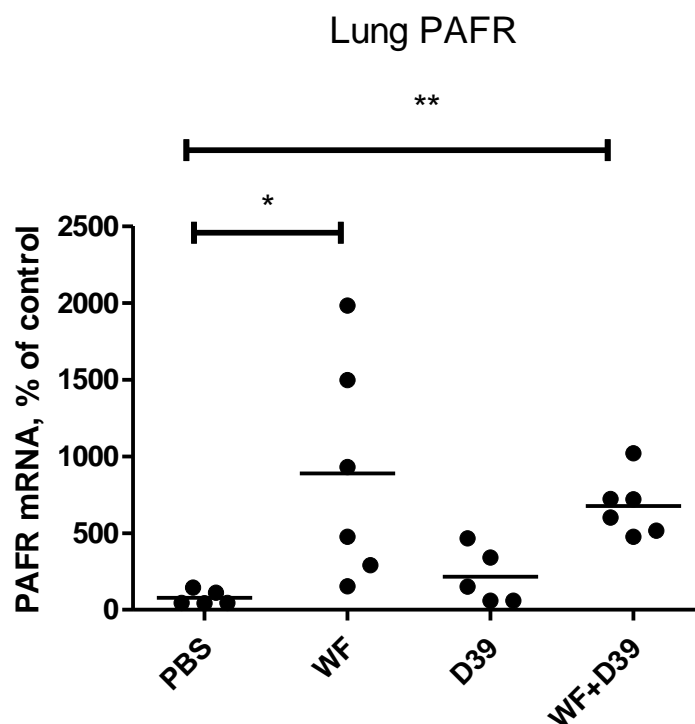


Figure 2: Exposure to welding fume alone and WF+bacteria caused a significant increase in PAFR mRNA expression compared to the PBS control (n=6). In infected mice, WF exposed mice had significantly higher PAFR mRNA expression compared to unexposed mice. Data are compared using Kruskal-Wallis test followed by Dunn's post-test.

4.2.2 Chronic exposure to WF by intranasal inoculation route, *S. pneumoniae* stain: D39

To model chronic exposure to welding fumes the following exposure regimen was used:

Monday, Wednesday, Friday of Week 1: either PBS or WF delivered intranasally under anaesthesia

Monday, Tuesday, Wednesday of Week 2: either PBS or WF delivered intranasally under anaesthesia

Thursday of Week 2: 5×10^6 *S. pneumoniae* delivered intranasally under anaesthesia. The number of pneumococci present in the inoculum was measured using quantitative bacterial culture.

Friday of Week 2: cull for analysis of CFU counts in blood, BALF and lungs and analysis of amounts of macrophages and neutrophils present in BALF. A tabular representation of the timing and type of intervention performed is shown below.

Day	Intervention
1	Intranasal inoculation of PBS/WF under anaesthesia
3	Intranasal inoculation of PBS/WF under anaesthesia
5	Intranasal inoculation of PBS/WF under anaesthesia
8	Intranasal inoculation of PBS/WF under anaesthesia
9	Intranasal inoculation of PBS/WF under anaesthesia
10	Intranasal inoculation of PBS/WF under anaesthesia
11	Intranasal inoculation of D39 under anaesthesia
12	Cull

For the chronic low dose exposure a total dose of 600µg/mouse of WF was delivered in 100µg/mouse instalments.

There were 6 mice/group. No pneumococci were seen in the blood. A significant increase in CFU counts was seen in the BALF (*p<0.05, figure 3A) and lungs (*p<0.05, figure 3B) of WF exposed mice compared to the controls. A significantly higher proportion of the cells recovered from the BALF were neutrophils in WF exposed mice compared to PBS exposed mice (**p<0.01, figure 3C).

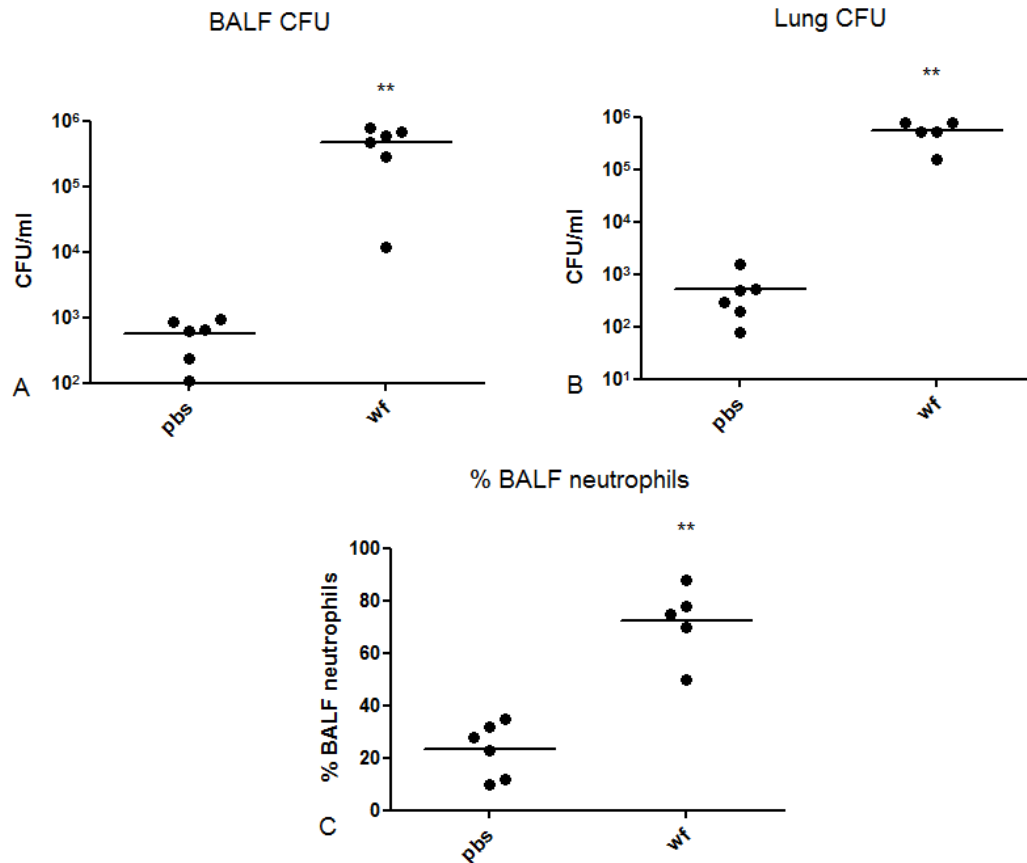


Figure 3: Chronic exposure to WF increases susceptibility to pneumococcal pneumonia. Exposure to WF significantly increased CFU counts in the (A) BALF and (B) lungs of WF exposed mice compared to the controls. (C) The proportion of BALF cells that were neutrophils were significantly higher in the WF mice compared to the controls. Data are from at least 5mice/group. Data are compared using Mann Whitney U test.

For the chronic high dose exposure a total dose of 3.6mg/mouse of WF was delivered in 600µg/mouse instalments.

There were 6 mice/group. No BALF was obtained for one WF exposed mouse due to a technical error. Further, another WF exposed mouse had to be excluded from the analysis due to no CFU counts being seen in either the BALF or the lungs. This

may be due to a technical error by which the mouse received no bacteria. The cytospin slides were of poor quality and could not be analysed.

No pneumococci were seen in the blood. A significant increase in CFU counts was seen in the BALF (* $p < 0.05$, figure 4A) and lungs (* $p < 0.05$, figure 4B) of WF exposed mice compared to the controls.

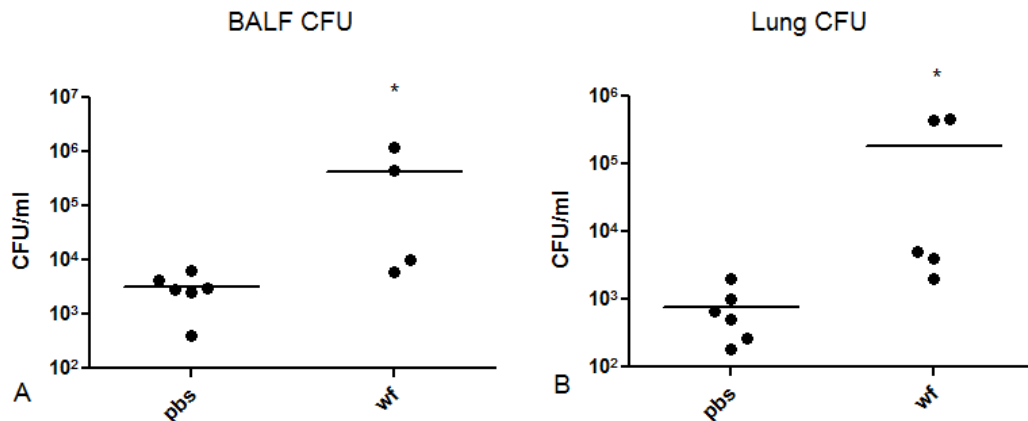


Figure 4: Chronic high dose exposure to WF increases susceptibility to pneumococcal pneumonia. Exposure to WF significantly increased CFU counts in the (A) BALF and (B) lungs of WF exposed mice compared to the controls. Data are from at least 5 mice/group. Data are compared using Mann Whitney U test.

4.2.3 Chronic exposure to WF using inhalation route; exposure conditions: whole body inhalation of stainless steel welding fumes for 10 days at a target concentration of 40mg/m^3 for 3 hours/day

Patti Erderly (Centre for Disease Control, USA) kindly agreed to mine stored lung mRNA from a mouse model of welding fume inhalation for this study. The data presented above showed that acute exposure to welding fumes in mice, via nasopharyngeal aspiration, caused an increase in pneumococcal infection and in PAFR mRNA. Hence, it was considered important to study PAFR mRNA expression in the mined samples. A description of the study design is outlined in the methods

chapter of this thesis. Briefly, mice (n=4/group) were exposed to stainless steel welding fume at a concentration of 40mg/m^3 for 3hours/day for 10 days. The mice were then culled and PAFR expression in the lung tissue was analysed. Taqman primers and probes were used for the RT-PCR reactions and the $\Delta\Delta\text{Ct}$ method was used for analysis of the data.

Study design and experimental procedures were conducted by Patti Ederly at the request of the author of this thesis. Data analysis was conducted by the author of this thesis.

PAFR mRNA expression was significantly increased (2-fold) 4hours (* $p<0.05$, figure 5A) and 28 days (** $p<0.01$, figure 5B) after the last exposure to welding fumes in exposed mice compared to control mice.

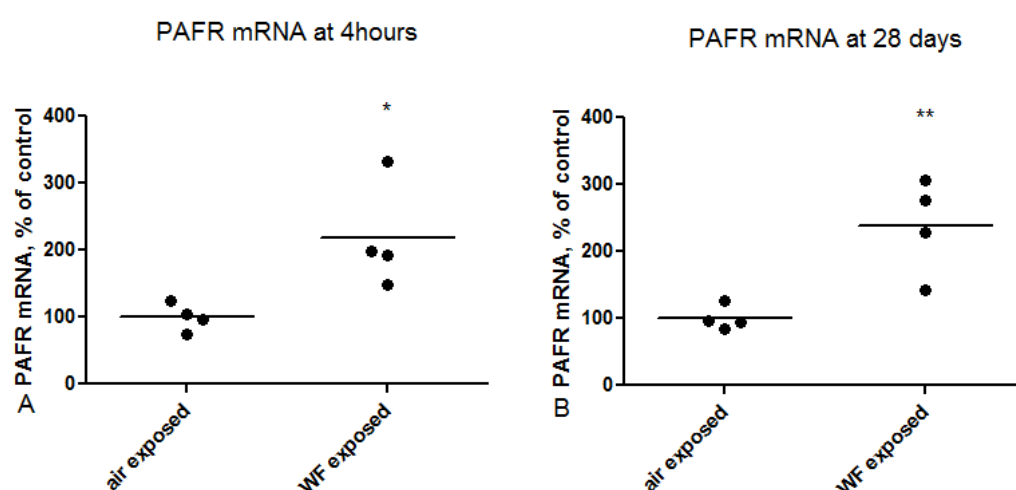


Figure 5: PAFR mRNA expression after the last exposure to stainless steel welding fumes in a 10day inhalation model of welding fume exposure in mice. A significant increase in PAFR mRNA expression was seen (A) 4 hours and (B) 28 days after the last exposure to WF compared to air exposed mice. The data are compared using unpaired t tests.

4.2.4 PAFR expression and localisation after WF exposure

Mice (n=3/group) were exposed to a total dose of 600µg/mouse of mild steel WF, as described in the methods chapter. The mice were then culled 24 hours later for lung immunohistochemical analysis. The images were scored by a blinded operator.

Immunohistochemical scores for alveolar+bronchial epithelial PAFR staining were higher for WF exposed mice compared to control mice (figure 6A). The difference was not significant ($p=0.11$). A qualitative observation of alveolar wall thickening, as indicated by the white arrows, was made in mice exposed to WF (figure 6C) which was not seen in the controls (figure 6B).

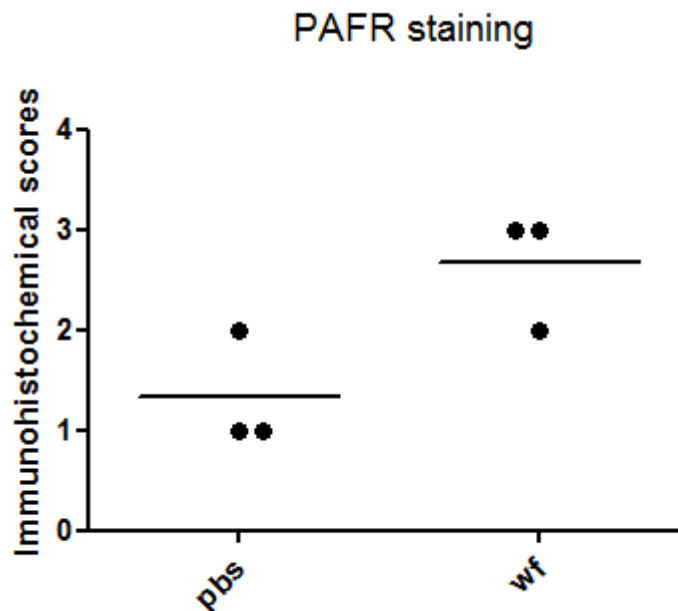


Figure 6A: Immunohistochemical analysis of mouse lungs after exposure to WF. WF exposed mice had higher immunohistochemical scores for alveolar+bronchial PAFR staining compared to control mice. This difference was not significant. The data are compared using a Mann-Whitney U test.

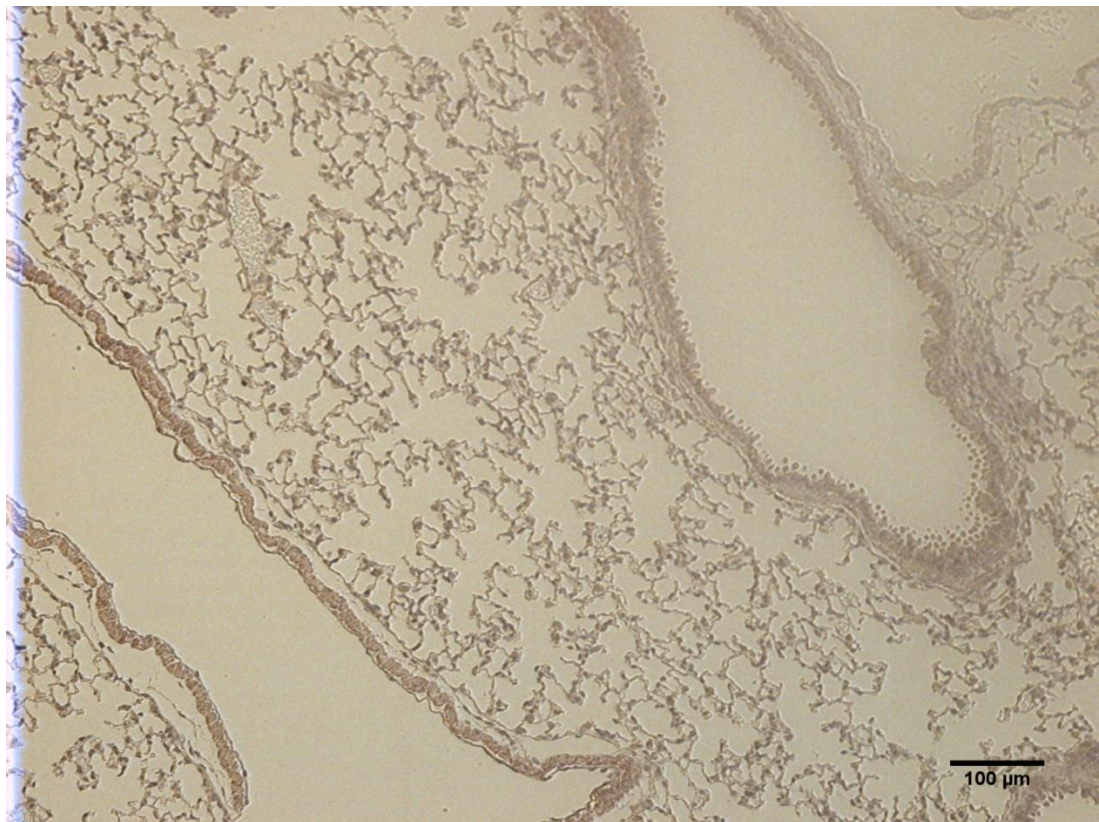


Figure 6B: PAFR staining seen in the unexposed mouse lungs.

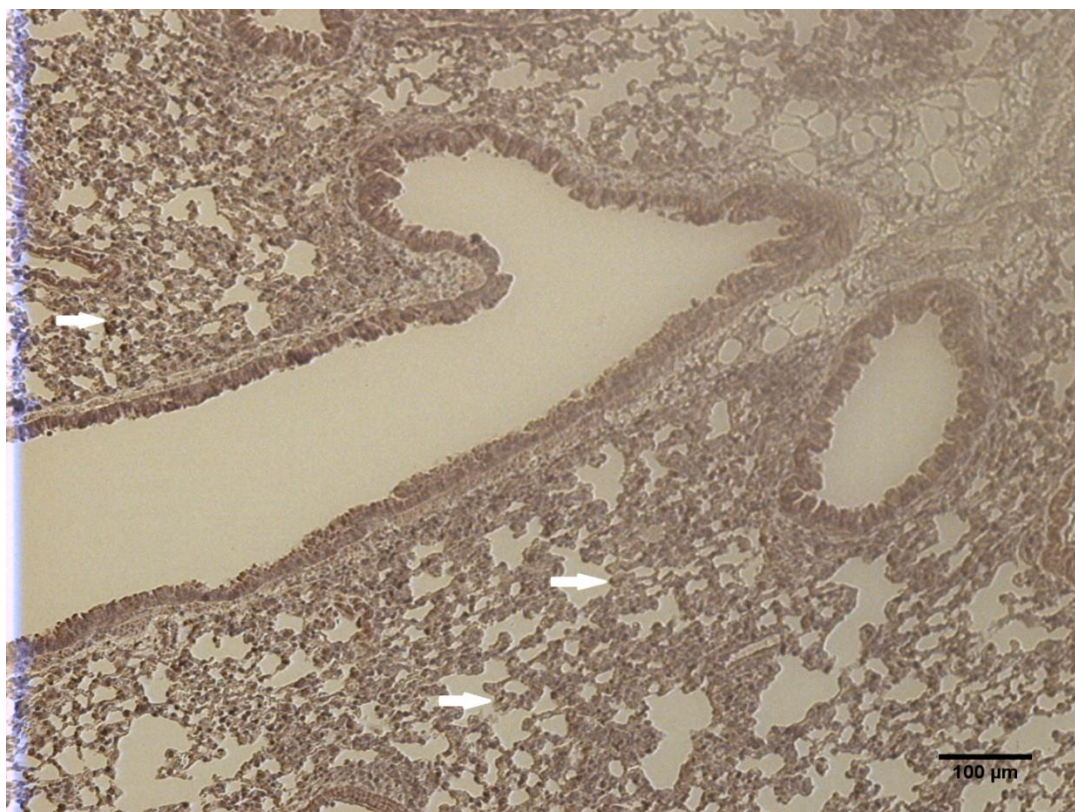


Figure 6C: PAFR staining seen in mouse lungs after exposure to WF. The white arrows indicate areas of alveolar wall thickening.

4.3 Conclusions

The data show, for the first time, that acute exposure to mild steel welding fume increases susceptibility of mice to pneumococcal infection in the lungs. This increase in infection is accompanied by an increase in PAFR mRNA expression which is consistent with the *in vitro* protein expression data presented in chapter 3. Further, the effect of chronic exposure to WF on susceptibility to pneumococcal infection was also studied. An increase in pneumococci in the lungs was seen in this exposure model. This is consistent with previous reports that chronic exposure to WF followed by infection with *L. monocytogenes* resulted in increased bacterial burdens compared to the controls in rats^{145,153}. These data provide first evidence of biological plausibility of susceptibility to pneumococcal infection after exposure to mild steel welding fumes.

In the intranasal instillation studies presented the pneumococcal stock used was not virulent enough to cause pneumococcal invasion into the blood. This may be a technical artefact caused by passages of the stock or long term storage at -80°C in nutrient broth containing 10% glycerol. However, this may also be because apart from exposure to WF there may be other risk factors that contribute to the development of invasive pneumococcal disease in welders. Indeed the limited epidemiological literature suggests that while welders are at a higher risk of developing lobar and bronchopneumonia, smoking is a major confounder in studying increased risk of welders to invasive pneumococcal disease^{4,142–144}. Hence, the data presented in this study suggest that exposure to welding fumes per se increases susceptibility to non-invasive pneumococcal disease in a mouse model.

A limitation of these studies is that the exposure was via intranasal inoculation rather than the physiological route which is inhalation. A dose of 600µg was the highest possible tolerated by a single mouse by intranasal inoculation under anaesthesia. The only other mouse study in which the WF was delivered in a similar way used a total dose of 400µg/mouse delivered in 100µg instalments over 4 different days¹³⁴. In the low dose chronic exposure experiment a total dose of 600µg/mouse was delivered in 100µg instalments over 6 different days. Other studies in rats have used a total dose of 2mg/rat^{139,145,155}. The U.S Environmental Protection agency recommends a daily exposure limit to particulate matter of 150µg/m³. However, little is known about the concentration of particulate deposits in the lungs after these exposures. Hence, estimating the dose to be delivered by nasopharyngeal aspiration in this mouse model is difficult. Thus, it was considered of importance to assess PAFR mRNA levels in WF exposed mice using a more relevant inhalation model.

In the welding fume inhalation mouse model, inhalation doses were set at 40mg/m³ for 3hours/day. After a 10 day exposure period it was found that PAFR mRNA levels

were increased in WF exposed mice compared to air exposed mice. PAFR levels were measured at both 4 hours and 28 days after the last exposure. The study may be extended to assess the earliest time point at which PAFR mRNA is upregulated and the longest time period over which it is sustained. This may provide clues about a window for PAFR-dependent enhanced susceptibility to pneumococcal infection in welders which is useful in a clinical context. Further, pneumococcal infection was not assessed in the inhalation model and would be interesting to assess in future work. Further work could also include the use of a PAFR knockout mouse to better understand the role of PAFR in welding fume-dependent increased susceptibility to pneumococcal pneumonia.

Immunohistochemical analysis of PAFR expression showed that the protein is expressed by the alveolar and bronchial epithelium. Quantifying expression levels showed that WF exposed mice had higher, although not significantly so, levels of PAFR in the alveolar and bronchial epithelium compared to control mice ($p=0.11$). Taken with the lung PAFR data, these data suggest that the higher PAFR mRNA levels seen in mice exposed to WF through both the instillation and inhalation routes may be localised to the bronchial and alveolar epithelium which are putative sites for pneumococcal infection. The functional studies using the PAFR blocker, CV-3988, in bronchial and alveolar epithelial cells, presented in chapter 3 of this thesis, provide functional relevance for these observations in mice. Additionally, alveolar wall thickening was also observed in mice exposed to WF compared to the controls. However, this observation could not be quantified.

Chapter 5: Viral-bacterial interactions in lower airway epithelial cells exposed to welding fume and potential therapies

5.1 Introduction and aims

Epidemiological studies have shown that occupational welders are more susceptible to viral pneumonia compared to the general population¹⁴³. However, little is known about the molecular mechanisms involved. Viral pneumonia is caused by viruses such as rhinoviruses (RVs) and respiratory syncytial virus^{156,157}. A known entry receptor for rhinoviruses in human cells is ICAM-1³².

A major cause of fatalities in patients with viral pneumonia, particularly vulnerable populations, is secondary bacterial infections. Data from previous chapters suggested that welders are more susceptible to pneumococcal pneumonia in a PAFR-dependent manner.

To date there are no effective preventative interventions or cures for rhinovirus infections. However, it has recently been shown that a sulphated galactose polymer derived from Rhodophyceae seaweed, iota-carrageenan (IC), has anti-rhinoviral properties, although the molecular mechanisms involved are unknown⁴¹. Further, randomised control trials in both adults and children showed that treatment with IC caused a reduction in rhinovirus levels in nasal washes^{39,40}. The compound has been deemed safe for topical applications and use in food by the U.S FDA. Iota carrageenan is also available as an over-the-counter nasal spray for treatment of viral colds down to 6 months of age. However, it remains unknown whether iota carrageenan is also effective against pneumococcal infections secondary to rhinovirus infections.

Hence, the aims of this study were:

- To assess ICAM-1-dependent increased susceptibility to rhinoviral infection in lower airway epithelial cells after exposure to welding fumes.
- To test whether exposure to welding fume followed by rhinoviral infection increases susceptibility to pneumococcal infection in these cells.

- To examine the role of PAFR in rhinoviral/pneumococcal interactions in welding fume exposed lower airway epithelial cells
- To study the effect of IC on rhinoviral/pneumococcal interactions in lower airway epithelial cells

5.2 Results

5.2.1 Exposure to mild steel welding fumes causes an upregulation of ICAM-1 mRNA

A549 and BEAS-2B cells were exposed to mild steel welding fume for 2 hours at the doses chosen based on the dose response data presented in chapter 3, i.e. 275 µg/ml for A549s and 200 µg/ml for BEAS-2Bs. ICAM-1 mRNA levels were assessed relative to the housekeeping gene, using TaqMan primers and probes. The data were analysed using the $\Delta\Delta C_t$ method.

Exposure of both A549s (78-fold, *** $p < 0.01$, figure 1A) and BEAS-2Bs (10-fold, *** $p < 0.01$, figure 1B) to mild steel welding fume caused a significant upregulation of ICAM-1 mRNA expression.

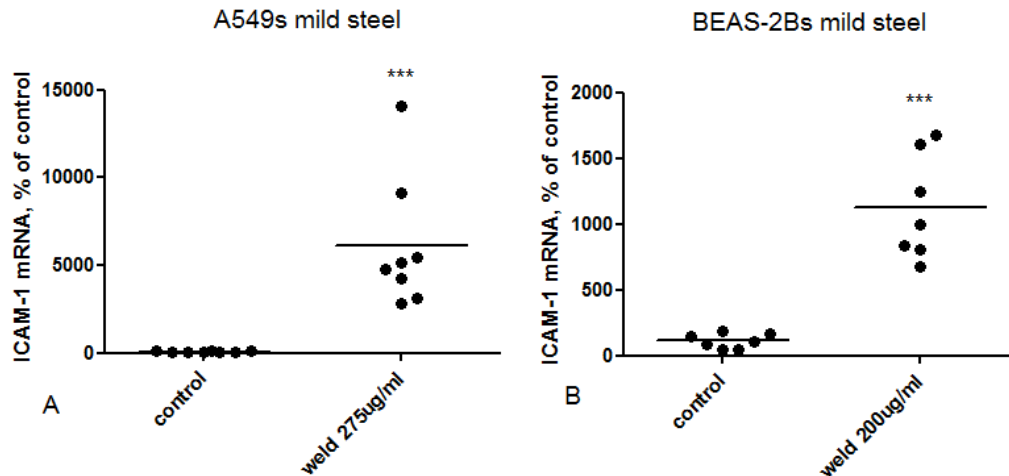


Figure 1: mRNA expression of ICAM-1 after exposure to mild steel welding fumes. Exposure caused an upregulation of ICAM-1 mRNA expression in (A) A549s (* $p < 0.05$), and (B) BEAS-2B cells (* $p < 0.05$). Data are from two separate experiments and are expressed as means of 4 samples per experiment. For each sample, mRNA from three separate wells was pooled for analysis. RT-PCR reactions were carried out in triplicate. Data are compared by unpaired t tests.

The cells were exposed to stainless steel WF at the same doses as those mentioned above for each cell line. ICAM-1 mRNA analysis was carried out as outlined above.

No difference in ICAM-1 mRNA levels was observed after exposure of the cells to stainless steel welding fume (figure 2).

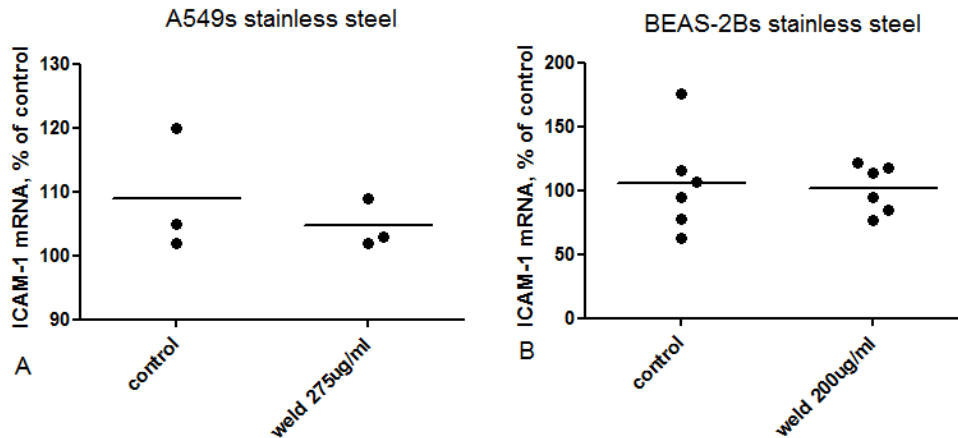


Figure 2: mRNA expression of ICAM-1 after exposure to stainless steel welding fumes. Exposure caused no change in ICAM-1 mRNA expression in (A) A549s or in (B) BEAS-2Bs. Data are from at least two separate experiments and are expressed as means of at least 2 samples per experiment. For each sample, mRNA from three separate wells was pooled for analysis. RT-PCR reactions were carried out in triplicate. Data are compared by unpaired t tests.

5.2.2 Exposure to welding fumes increases rhinoviral infectivity of A549s and BEAS-2Bs

Previous data showed that mild steel exposure increases susceptibility of lower airway epithelial cells to rhinoviral infection by causing an upregulation of the rhinoviral entry receptor, ICAM-1. However, no such upregulation was observed after exposure of the cells to stainless steel WF. Hence, it was important to study the effect of exposure of the cells to WF on rhinoviral infection of lower airway epithelial cells.

A549 and BEAS-2B cells were exposed to the pre-stated doses of WF and then infected with a rhinovirus, RV-16, at an MOI of 1. The amount of rhinoviral mRNA present in the cells was measured using quantitative real-time PCR. The data were normalised to the housekeeping gene and analysed using the $\Delta\Delta C_t$ method. UV

inactivated virus and media in which the virus was grown but from which the virions were filtered out were used as controls for viral entry into the cells.

Exposure of A549s and BEAS-2Bs to mild steel and stainless steel welding fumes causes significantly increased rhinoviral infection of these cells (figure 3). A 2-fold increase in the amount of viral mRNA seen in A549 cells was seen after exposure to mild steel welding fume. In BEAS-2Bs a 3-fold increase was seen. However in both A549s and BEAS-2Bs exposed to stainless steel welding fumes a 4-fold increase in the amount of viral mRNA was seen.

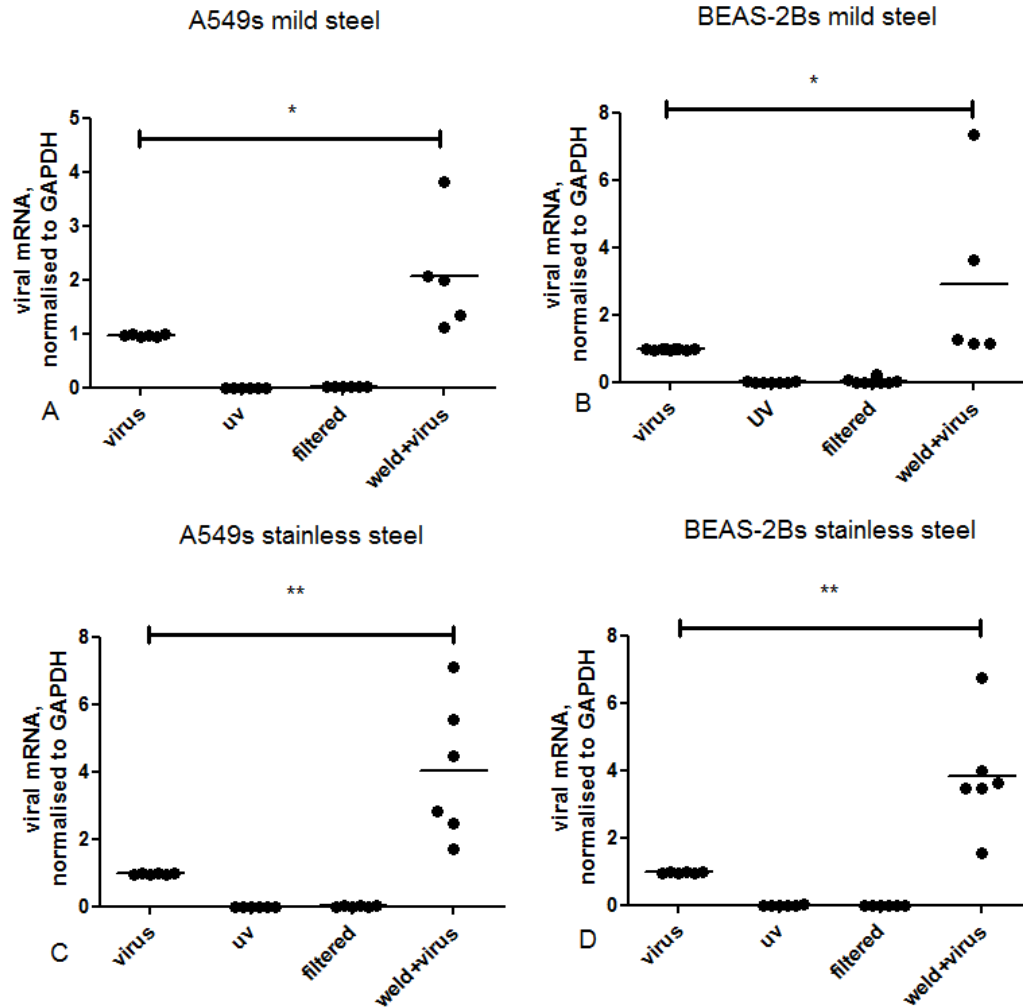


Figure 3: Exposure of lower airway epithelial cells to welding fume following rhinoviral infection in order to assess viral infectivity of these cells. A significant increase in the amount of viral RNA present in (A) A549 cells exposed to mild steel WF (* $p < 0.05$), (B) BEAS-2B cells exposed to mild steel WF (* $p < 0.05$) cells, (C) A549 cells exposed to stainless steel WF (** $p < 0.01$) and (D) BEAS-2B cells exposed to stainless steel WF (** $p < 0.01$). Data are from at least two separate experiments. In each experiment mRNA from 3 samples per condition was analysed. Data are expressed as means and are compared using unpaired t tests.

5.2.3 Exposure to welding fumes increases susceptibility of rhinovirus infected A549s and BEAS-2Bs to bacterial adhesion and invasion

A549 and BEAS-2B cells were exposed to WF and then infected with RV-16. Secondary pneumococcal infection was then assessed as described in the methods chapter.

Exposure of A549s and BEAS-2Bs to welding fume followed by infection with RV-16 caused a significant increase in pneumococcal adhesion to and invasion of these cells (figure 4). In all experiments a significant increase in pneumococcal adhesion and invasion was seen in virus infected cells compared to the control and in welding fume exposed cells compared to the control.

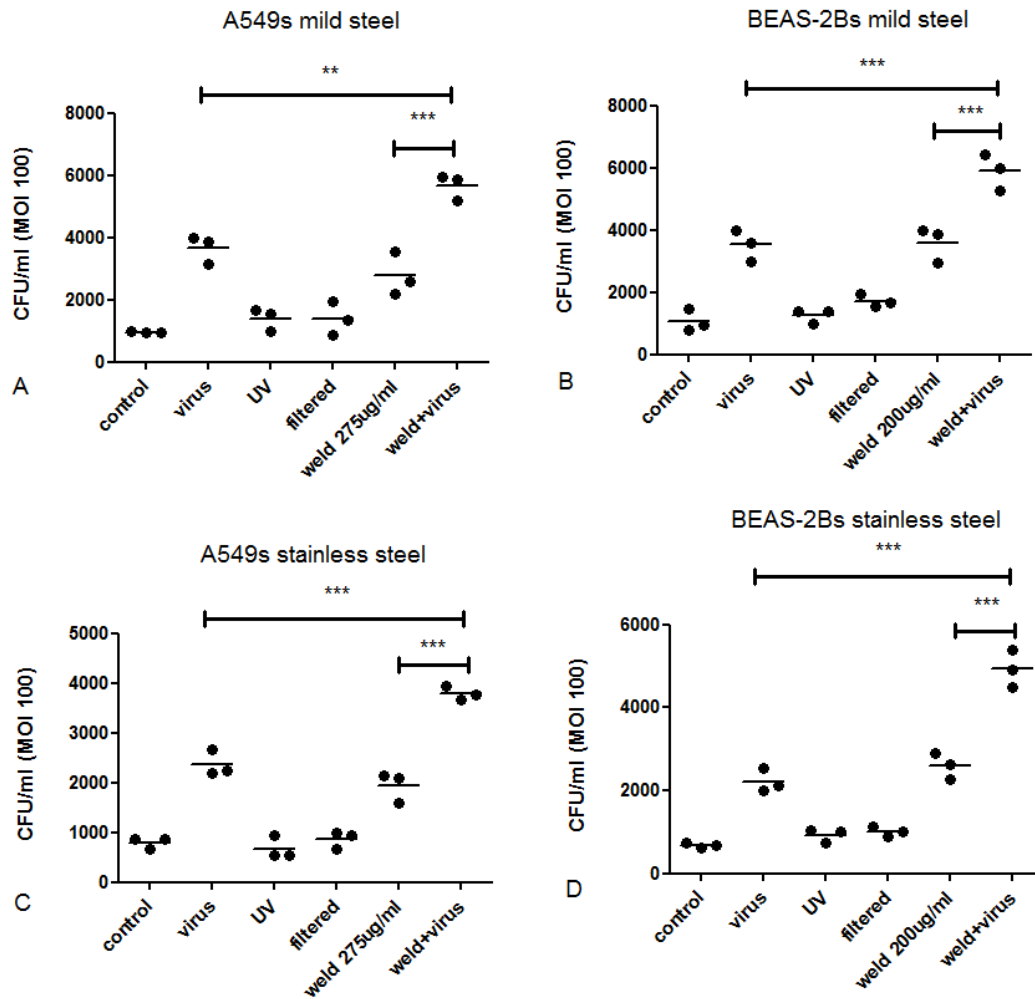


Figure 4: Exposure of lower airway epithelial cells to welding fume followed by viral infection, in order to assess secondary pneumococcal infection. A significant increase in secondary bacterial infection, compared to either cells only infected with virus or only exposed to WF, was seen in (A) A549s exposed to mild steel WF, (B) BEAS-2Bs exposed to mild steel WF, (C) A549s exposed to stainless steel WF and (D) BEAS-2Bs exposed to stainless steel WF. Data are from at least 3 separate experiments. Each data point represents one experiment and is the mean of data from at least 3 separate wells. Data are compared using 1 way ANOVA followed by Bonferroni's multiple comparison tests.

In order to assess the effect of chronic exposure to welding fume in a similar system the cells were exposed to each type of WF at a concentration of 10µg/ml overnight. This dose was chosen because it was the highest dose which the cells could be exposed to overnight without a loss in cell adherence to the tissue culture plates or changes in cell morphology being observed.

Exposure of A549 and BEAS-2B cells to a low dose of welding fume overnight, followed by infection with RV-16 also caused a significant increase in pneumococcal adhesion and invasion (figure 5). Overnight exposure of A549 cells to both mild steel and stainless steel WF caused a significant increase in pneumococcal adhesion and invasion of these cells (**p<0.01, figure 5). In BEAS-2B cells, exposure to mild steel WF caused a 1.8 fold increase in pneumococcal adhesion and invasion (figure 5B) but this was not statistically significant. However, exposure to stainless steel WF caused a significant increase in pneumococcal adhesion and invasion in these cells (**p<0.01, figure 5D). In all experiments a significant increase in bacterial infection was seen in virus infected cells compared to the control.

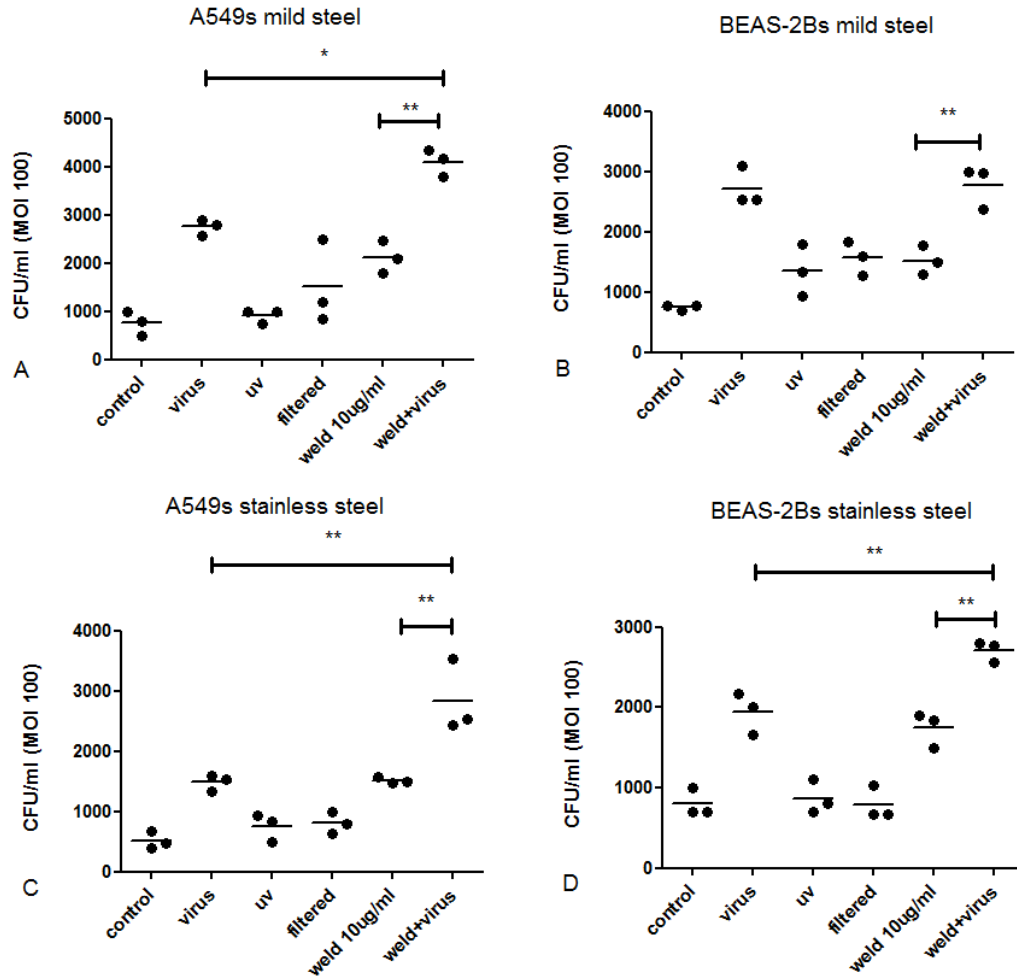


Figure 5: Exposure of lower airway epithelial cells to low doses of welding fume overnight followed by viral infection, in order to assess secondary pneumococcal infection. A significant increase in secondary bacterial infection compared to either cells only infected with virus or only exposed to WF, was seen in (A) A549s exposed to mild steel WF and infected with rhinovirus, and (B) A549s exposed to stainless steel WF and infected with rhinovirus. In (C) BEAS-2Bs exposed to mild steel WF, no difference in CFU/ml was seen in cells exposed to mild steel WF and infected with virus compared to those only infected with virus. However, a significant increase in secondary bacterial infection was seen when cells exposed to mild steel WF and infected with virus were compared to those only exposed to mild steel WF (** $p < 0.01$). In (D) BEAS-2Bs exposed to stainless steel WF and infected with RV-16, a significant increase in bacterial infection compared to either cells only infected

with virus or only exposed to WF was seen. Data are from at least 3 separate experiments. Each data point represents one experiment and is the mean of data from at least 3 separate wells. Data are compared using 1 way ANOVA followed by Bonferroni's multiple comparison tests.

5.2.4 Viral- and WF+viral-stimulated increased pneumococcal adhesion and invasion of A549 and BEAS-2B cells is PAFR-dependent

Previous data has shown that rhinoviral infection of lower airway epithelial cells and exposure to WF followed by rhinoviral infection increases susceptibility of these cells to secondary pneumococcal infections. This led to the question: is this increased susceptibility PAFR-dependent?

In order to assess this the cells were exposed to welding fumes, infected with RV-16 and pre-treated with the PAFR blocker, CV-3988, before pneumococcal infection. Since similar effects of both types of WF was seen on virus-induced pneumococcal adhesion and invasion in both cell types, only mild steel WF was used to confirm the role of PAFR using CV-3988.

CV-3988 attenuated both rhinovirus induced and WF+rhinovirus induced pneumococcal invasion and adhesion in both cell types (figure 6). In all experiments rhinoviral infection alone and exposure to WF followed by rhinoviral infection caused a significant increase in secondary pneumococcal infection compared to the controls. However, in A549 cells no difference in pneumococcal adhesion and invasion was seen between cells infected with rhinovirus only and cells exposed to WF followed by rhinovirus infection (figure 6A). In BEAS-2B cells there was a 1.4 fold difference in secondary pneumococcal infection between these two conditions, which was not statistically significant (figure 6B). This was not consistent with data presented in figure 4 of this chapter.

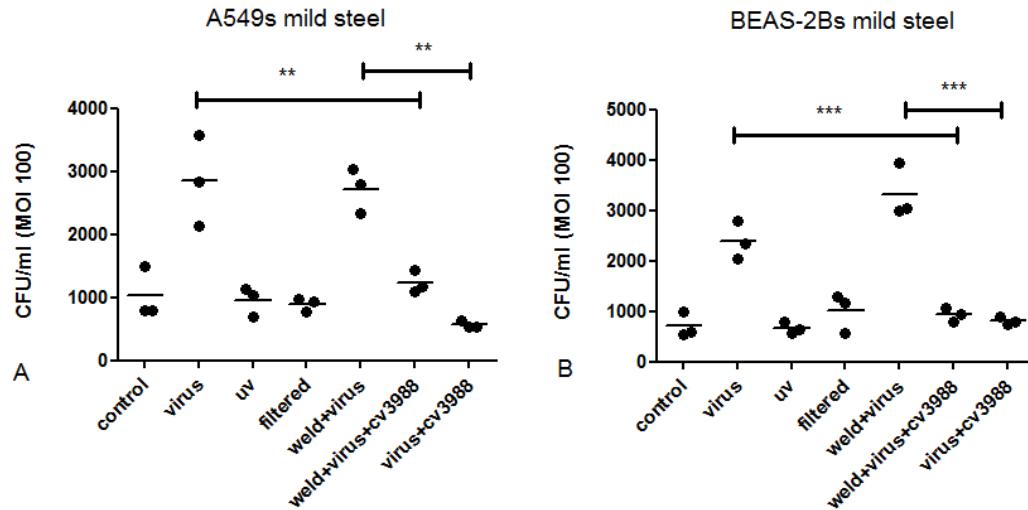


Figure 6: Use of the PAFR blocker, CV-3988, to assess the role of PAFR in rhinoviral- and WF+rhinoviral-induced pneumococcal adhesion and invasion. RV-16- and mild steel WF+RV-16-stimulated secondary pneumococcal infection was attenuated by CV-3988 in (A) A549, and (B) BEAS-2B cells. Data are from 3 separate experiments. Each data point represents one experiment and is the mean of data from 3 wells. Data are compared using Bonferroni's multiple comparison tests.

5.2.5 Treatment with iota carrageenan attenuates rhinovirus-induced secondary pneumococcal infections in lower airway epithelial cells

With the current lack of treatments for rhinovirus infections it was of interest to investigate the anti-rhinoviral effects of a recent compound of interest, iota carrageenan. Specifically, it was of interest to investigate whether iota carrageenan affects rhinovirus-induced secondary pneumococcal infections.

Alveolar and bronchial epithelial cells were treated with 25µg/ml iota carrageenan during rhinovirus infection. After removal of the virus the iota carrageenan was added back to the cells and left overnight. The cells were then infected with *S. pneumoniae* at an MOI of 100. Adherent and internalised bacteria were then assessed.

In both A549s (** $p < 0.001$, figure 7A) and BEAS-2BS (** $p < 0.001$, figure 7B) IC attenuated rhinovirus-induced pneumococcal infections. The rhinovirus infection induced a 2 fold increase in pneumococcal infection in both A549s and BEAS-2Bs (** $p < 0.001$, figure 7).

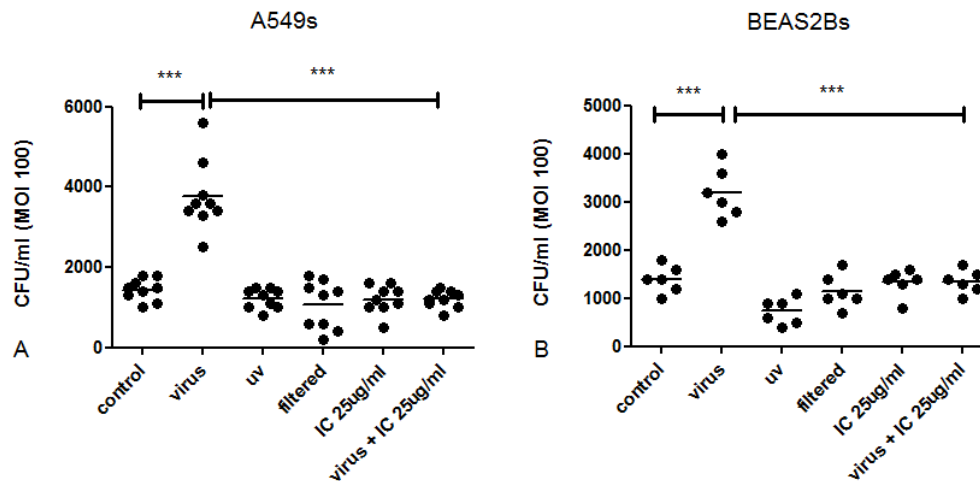


Figure 7: Effect of IC on rhinovirus-induced pneumococcal infections in (A) A549 cells and (B) BEAS-2B cells. In both cell types rhinovirus infection significantly increased pneumococcal infection which was reduced to control levels by treatment with IC. The data are from at least 2 experiments. Each data point represents a single replicate. The data are compared using 1 way ANOVA followed by Bonferroni's multiple comparison test.

5.3 Conclusions

Welders are more susceptible to viral pneumonia compared to the general population¹⁴³. Hence, one of the aims of this study was to assess the effect of exposure to welding fumes on the susceptibility of lower airway epithelial cells to rhinoviral infection. ICAM-1 is a known receptor used by rhinoviruses for entry into lower airway epithelial cells. Surface and mRNA expression has been shown to increase after exposure of A549 cells to RV-16 in a virus dose dependent manner with expression peaking at MOI 1. RV-16 infection (MOI 1) has also been shown to

increase ICAM-1 surface expression in primary bronchial cells³³. In this investigation ICAM-1 mRNA was analysed. In a preliminary experiment conducted to test the viability of the virus it was observed that constitutive expression of ICAM-1 was low relative to expression after exposure of both A549 and BEAS-2B cells to rhinovirus. This is consistent with what is reported in the literature³³. In order to assess whether exposure to welding fume increases susceptibility of lower airway epithelial cells to infection by RV-16, ICAM-1 mRNA was compared in cells exposed or unexposed to welding fumes. In both cell types a significant increase in ICAM-1 mRNA was observed after exposure to mild steel welding fume. It has previously been reported that exposure of rat lung epithelial cells to diesel exhaust particles causes increased ICAM-1 mRNA expression¹⁴⁷. However, this is the first time that increased ICAM-1 mRNA expression has been observed in human respiratory epithelial cells exposed to mild steel WF. ICAM-1 mRNA expression was not affected by exposure to stainless steel welding fume.

In order to further address the question of susceptibility to infection by rhinovirus, viral infectivity of the cells was assessed. This was done by measuring the amount of viral mRNA present in the cells after infection. The data showed that lower airway epithelial cells exposed to both mild steel and stainless steel welding fume and then infected with RV-16 contained approximately 2-4 times more viral RNA compared to those unexposed to WF and also infected with RV-16. This suggests that while exposure to stainless steel WF increases susceptibility to rhinoviral infection, this increased susceptibility may not be ICAM-1 dependent as is the case with exposure to mild steel welding fume. Members of the low density lipoprotein receptor (LDLR) family have also been implicated as entry receptors for rhinoviruses³³. Further work is required to better understand the molecular mechanisms involved in stainless steel WF exposure-conferred increased susceptibility of lower airway epithelial cells to rhinoviral infection.

Since welders are more susceptible to viral and pneumococcal pneumonia and secondary pneumococcal infections can lead to fatalities in patients with viral pneumonia it was important to assess the effect of exposure to welding fumes and viral/bacterial infections in lower airway epithelial cells. In order to do this both acute and chronic exposure to welding fumes was modelled *in vitro*. Acute, short-term exposure to WF, as referred to in chapter 3, followed by rhinoviral infection caused increased pneumococcal adhesion and invasion of lower airway epithelial cells. Viral stimulation of pneumococcal adhesion and invasion was similar to WF stimulation of pneumococcal infection in these experiments.

Chronic exposure conditions were modelled using a 24hour stimulation of the cells with WF. A dose of 10µg/ml was used as it was the highest dose that was tolerated by the cells for 24 hours. Under these exposure conditions pneumococcal adhesion and invasion was stimulated above control levels in A549s for both types of WF but in BEAS-2Bs for only stainless steel WF. No additive effect of WF stimulation and rhinoviral infection was seen in BEAS-2B cells exposed to mild steel WF. These data suggest that chronic exposure to higher doses of mild steel WF may be required for increased pneumococcal infection of these cells. While chronic exposure to higher doses is difficult to model in this system due to issues with cell viability mentioned previously, it is not unrealistic to expect under physiological conditions. Hence, for further studies chronic exposure may be better modelled using an *in vivo* system. An additive effect of WF stimulation and rhinoviral infection on pneumococcal adhesion and invasion was observed in both A549 cells exposed to both types of WF and in BEAS-2B cells exposed to stainless steel WF.

Data presented in chapter 3 showed a role for PAFR in WF-stimulated pneumococcal adhesion and invasion of lower airway epithelial cells. Additionally, it has been previously shown that human pharyngeal and tracheal epithelial cells either exposed to poly I:C or infected with human rhinovirus have increased PAFR

expression and are more susceptible to pneumococcal adhesion. Increased pneumococcal adhesion was attenuated by PAFR blockers in both these studies^{30,43}. Hence, it was important to assess whether PAFR has a role in rhinovirus-stimulated pneumococcal infection of alveolar and bronchial cells, which are putative sites of pneumococcal infection. The PAFR blocker, CV-3988, attenuated both RV-16-stimulated pneumococcal infection and WF+RV-16-stimulated pneumococcal infection in A549 and BEAS-2B cells. This further strengthens the case presented in chapter 3 for PAFR as an anti-infective target in welders. In these experiments the CFU counts observed after exposure of the cells to mild steel WF followed by rhinoviral infection were approximately 2 fold lower compared to those presented in figure 4 of this chapter. Further, no additive effect of RV-16 and WF was observed on pneumococcal adhesion and invasion in A549 cells. This may be due to reduced virulence of the pneumococcal stocks, caused by long term storage. Similar effects on pneumococcal virulence have been previously observed in the lab.

In this study the effect of IC on rhinovirus-induced pneumococcal infection was also assessed. IC has recently been examined for anti-rhinoviral activity³⁹⁻⁴¹. However, to date it is unknown whether this anti-rhinoviral activity extends to an effect on secondary bacterial infections. Hence, this study provides first evidence that IC attenuates secondary pneumococcal infections after a rhinovirus infection. Currently there are no effective preventions or treatments for rhinovirus infections. Hence, the known anti-rhinoviral activity of IC, its safety profile and the evidence presented in this chapter for its protective effect on secondary pneumococcal infections make it a good candidate for further investigation as an anti-rhinoviral therapy. However, further work needs to be done to better understand the mechanisms by which IC exerts its anti-rhinoviral effects.

Chapter 6: PAFR expression and localisation in the human lung after exposure to airborne particulate matter

6.1 Introduction and aims

PAFR is a known entry receptor for *S. pneumoniae*⁸. The data presented in chapters 3 and 4 of this thesis and previous work in our lab have shown that exposure to WF and cigarette smoke extract (CSE) causes a PAFR-dependent increase in pneumococcal adhesion and invasion of lower airway epithelial cells *in vitro*. Additionally, higher PAFR expression has been shown in the bronchial epithelium of smokers compared to healthy controls¹²³. Chronic obstructive pulmonary disorder (COPD) is a chronic respiratory condition of which one of the causes is smoking⁶⁰. Hence PAFR expression and localisation in these subjects was also of interest.

COPD patients are highly susceptible to infections. Rhinoviruses have been shown to be associated with COPD exacerbations and patients with a rhinovirus infection and secondary bacterial infections such as pneumococcal infections have increased airway inflammation and poorer clinical outcomes⁶⁰. Data presented in chapter 5 showed that PAFR mediates rhinovirus induced pneumococcal infection of lower airway epithelial cells. Hence, PAFR expression and localisation in human subjects experimentally infected with rhinovirus was of interest to study.

Endobronchial biopsies were obtained from healthy non-smokers, smokers without airway obstruction, and smokers with COPD, before and after infection with rhinovirus RV-16¹⁵⁸.

The aims of this study were to assess the localisation of PAFR protein in human lung samples from a welder compared to a healthy control. Additionally, PAFR mRNA expression and protein expression and localisation were also assessed in the endobronchial biopsies of smokers and smokers with COPD from an experimental rhinovirus infection model.

6.2 Results

6.2.1 PAFR expression and localisation in the lungs of welders

Sophie Lanone (INSERM, France) kindly provided paraffin embedded lung biopsy section slides from a non-smoking control and a non-smoking welder for immunohistochemical analysis. While the functional relevance of PAFR expression to vulnerability to pneumococcal infections has been presented in chapter 3, these slides allowed the characterisation of PAFR expression and distribution in a welder's lung. Hence, although the number of samples available made it impossible to carry out statistical analyses they provide some valuable insights.

Image analysis of these slides was conducted as described in the methods chapter of this thesis. A mouse anti-human PAFR antibody was used to stain the tissue (brown stain). The nuclei were visualised using 3, 3' diaminobenzidine (DAB) (blue stain). A qualitative assessment of PAFR positivity was carried out. More PAFR positive bronchial epithelial cells, indicated by the black arrows, were seen in the welder sample (figure 1B) compared to the non-smoking control (figure 1A). Additionally, large amounts of particulate matter deposits were observed in the welder sample as indicated by the white arrows. Such deposits were absent in the control sample.

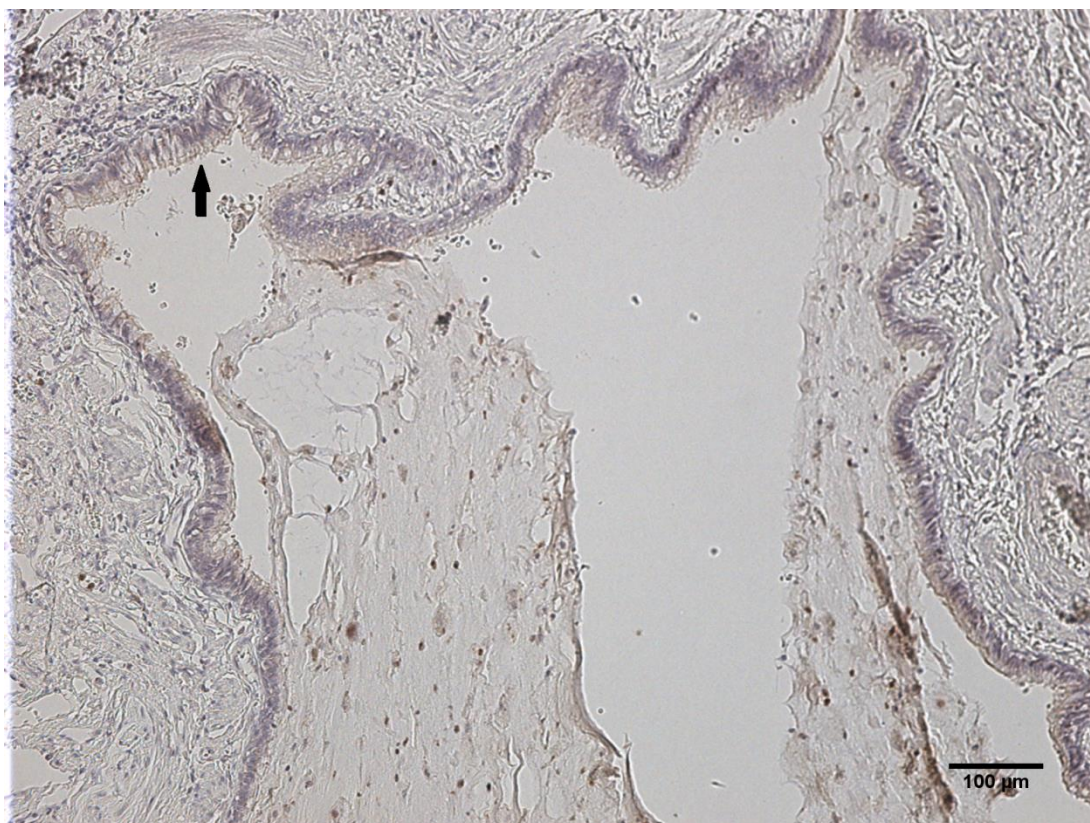


Figure 1A: PAFR staining seen in the non-smoking control. Staining is seen on the bronchial epithelium, which showed normal pathology, as indicated by the black arrow. No particulate matter deposits were seen in this sample.

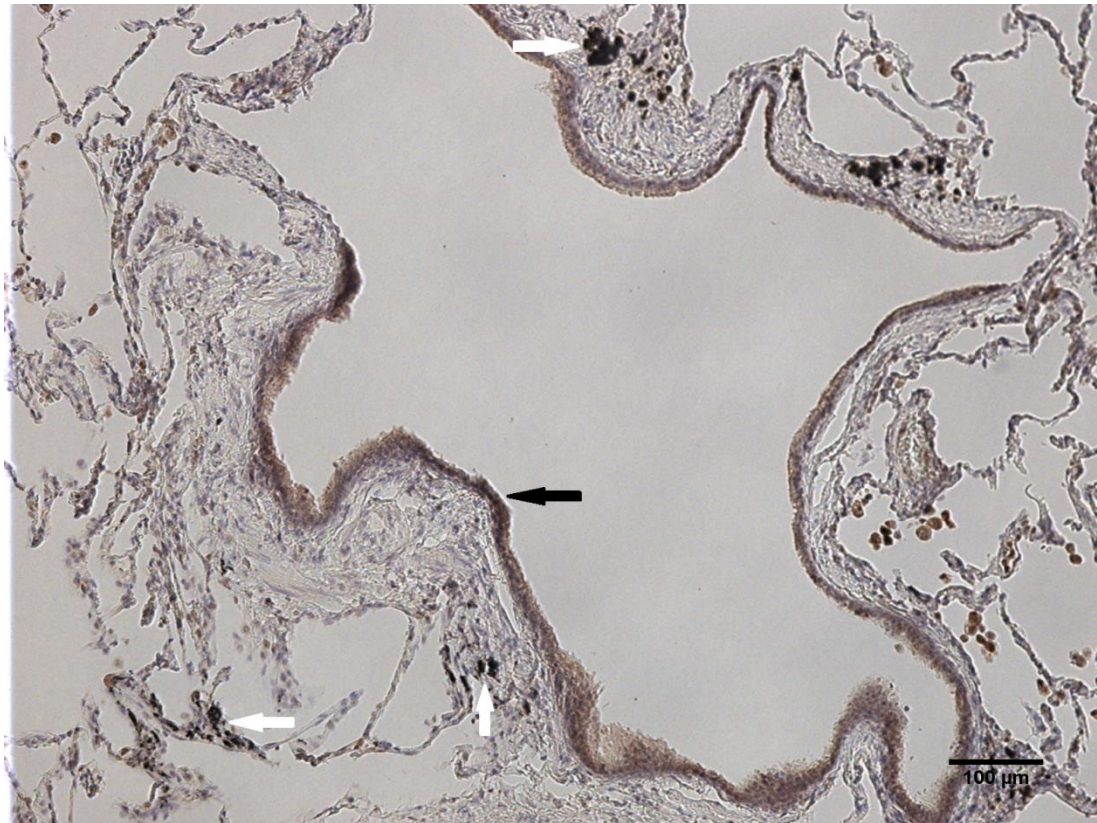


Figure 1B: PAFR staining seen in the non-smoking welder sample. Staining is seen on the bronchial epithelium, which showed normal pathology, as indicated by the black arrow. The white arrows indicate particulate matter deposits seen in the sample.

6.2.2 Human PAFR expression in an experimental model of rhinovirus infection in smokers and smokers with COPD

RNA extractions, cDNA synthesis and RT-PCR reactions were carried out by a technician in the laboratory, Lee Koh. Experimental design and data analysis were carried out by the author of this thesis.

6.2.3 Smokers with COPD have higher baseline levels of PAFR mRNA compared to smokers without COPD and non-smokers

The subject demographics for the study are included in table 1. There was no significant difference in age or body mass index between the three groups.

Table 1. Subject Demographics and endobronchial PAFR mRNA expression

	Non-smokers	Smokers	COPD
N	18	16	14
Age (yr)	59.2 ± 1.6	54.3 ± 1.8	59.6 ± 2.2
Body mass index (kg/m ²)	25.2 ± 1.0	27.1 ± 0.9	25.7 ± 1.1
Females (n)	9	7	5
Smoking pack years (n)	0	33.25 ± 2.21	39.35 ± 2.24
FEV ₁ (% predicted)	112.3 ± 4.4	102.9 ± 3.4	64 ± 3.4
FVC (% predicted)	124.4 ± 4.8	117.5 ± 3.0	95.0 ± 3.5
FEV ₁ /FVC	78 ± 1.0	75 ± 1.0	62 ± 1.7

Table 1: Subject demographics for the study. Data are summarised as mean ± standard error of mean. FEV₁: forced expiratory volume in 1 s, FVC: forced vital capacity

Baseline PAFR mRNA expression levels were compared between non-smokers, smokers and smokers with COPD. Since exposure of lower airway epithelial cells to CSE caused a PAFR-dependent increase in pneumococcal adhesion and invasion

of these cells it was hypothesised that smokers and smokers with COPD have higher baseline expression levels of PAFR.

Baseline PAFR mRNA expression levels were assessed in 18 non-smokers, 16 smokers and 14 smokers with COPD using TaqMan primers and probes. Relative expression levels were measured and normalised to the housekeeping gene, GAPDH.

Baseline expression levels in smokers and smokers with COPD were significantly higher compared to non-smokers (figure 2). No difference in levels was seen between smokers and smokers with COPD.

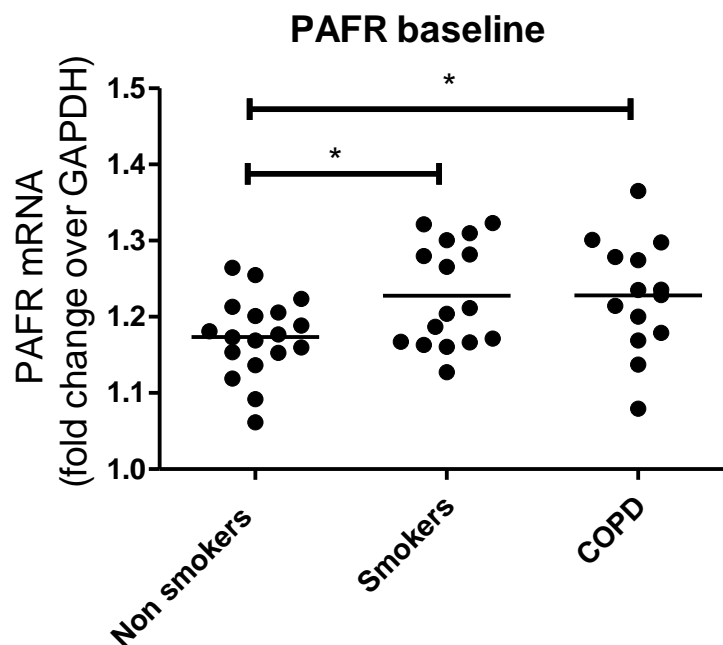


Figure 2: Baseline PAFR mRNA expression in non-smokers, smokers and smokers with COPD. Real time PCR reactions were carried out in triplicate. Significantly higher levels were seen in smokers and smokers with COPD compared to the non-smoking controls (* $p < 0.05$). Data are compared using 1 way ANOVA followed by Dunnett's multiple comparison test.

6.2.4 Rhinovirus infection does not increase PAFR mRNA expression in the human lung

Based on the data presented in chapter 5 which showed that rhinoviral infection of lower airway epithelial cells causes PAFR-dependent increased pneumococcal infection it was hypothesised that rhinoviral infection upregulates PAFR expression. To assess this, baseline PAFR mRNA levels were compared with 7 day post-rhinoviral infection levels. Paired samples were available from 9 non-smokers, 8 smokers and 7 smokers with COPD. The day 7 samples were analysed and processed similarly to the baseline samples, as outlined above.

Paired analyses showed no significant difference in PAFR mRNA expression before and after rhinovirus infection in non-smokers with a trend towards higher PAFR expression after infection ($p=0.09$) (figure 3A). There was no difference in expression, before and after infection, in either smokers or smokers with COPD (figure 3B, 3C).

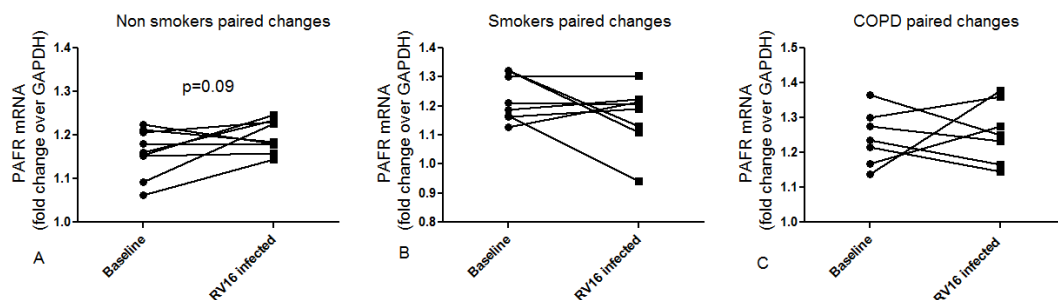


Figure 3: Paired changes in PAFR expression before and after rhinovirus infection in (A) non-smokers, (B) smokers and (C) smokers with COPD. A trend towards higher mRNA expression of PAFR was seen in non-smokers after rhinoviral infection. No difference in PAFR mRNA expression was seen in either smokers or smokers with COPD before and after rhinovirus infection. Data are compared using paired t tests.

6.2.5 PAFR mRNA expression in non-smokers, smokers and smokers with COPD 7 days after rhinovirus infection.

Although PAFR mRNA expression did not change within each group of subjects after rhinovirus infection, it was of interest to compare post-infection PAFR mRNA levels between groups. This was done to assess whether smokers with COPD are at a higher risk of PAFR-mediated pneumococcal infection compared to non-smokers after rhinovirus infection.

No difference in post-rhinoviral infection PAFR mRNA levels was seen between non-smokers and smokers with COPD though there was a trend towards higher levels in smokers with COPD ($p=0.08$). Further, a trend towards higher levels in smokers with COPD was seen compared to smokers without reduced lung function ($p=0.09$).

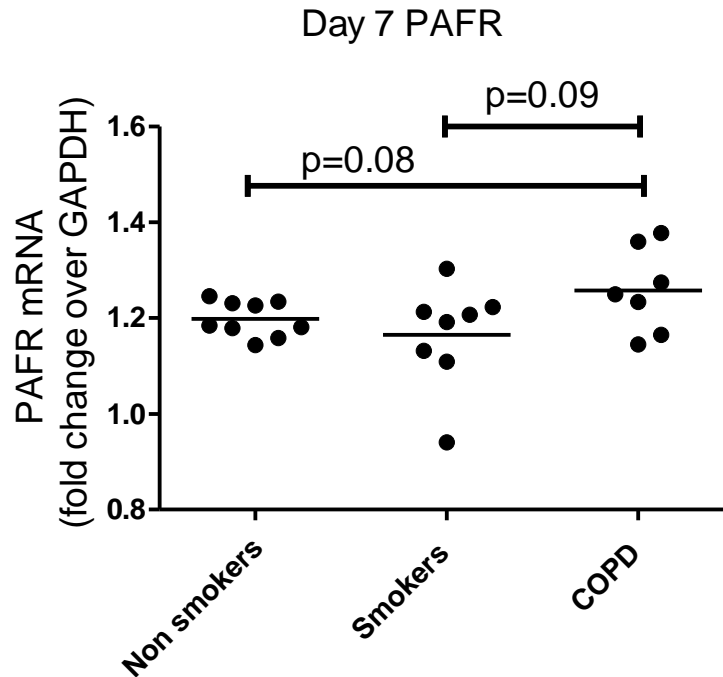


Figure 4: Comparison of post-rhinoviral infection PAFR mRNA levels in non-smokers, smokers and smokers with COPD. A trend towards higher expression levels was seen in smokers with COPD compared to non-smokers and smokers with normal lung function. Data were compared using t tests.

6.2.6 PAFR positive cells are predominantly located at the bronchial air-tissue interface

Paraffin embedded baseline bronchial tissue was used to study the localisation of PAFR protein expression using immunohistochemical methods. A mouse anti-human PAFR antibody was used to stain the tissue (brown stain). The nuclei were visualised using 3,3' diaminobenzidine (DAB) (blue stain).

Baseline tissue samples were available from 7 non-smokers, 7 smokers and 7 smokers with COPD. Due to the limited availability of samples it was not possible to quantify and compare cell specific PAFR expression. However, it was possible to carry out a qualitative analysis of the samples available.

Platelet-activating factor receptor immunostaining showed positive cells in the airway epithelium of all 21 subjects. Representative images of the samples available for analysis are shown below (figure 5). PAFR positive goblet cells were found in both smokers and smokers with COPD (figure 5C). PAFR positive bronchial submucosal gland cells, as seen in figure 5D, were also found in all 10 subjects where bronchial glands were sampled by the biopsy (controls n=4, smokers n=3, and smokers with COPD n=3). PAFR positivity of other cells, such as smooth muscle cells was minimal to absent (figure 5A). Areas of squamous metaplasia were found only in smokers and smokers with COPD. PAFR immunostaining of squamous metaplasia was lower than areas of more normal bronchial epithelium (figure 5B).

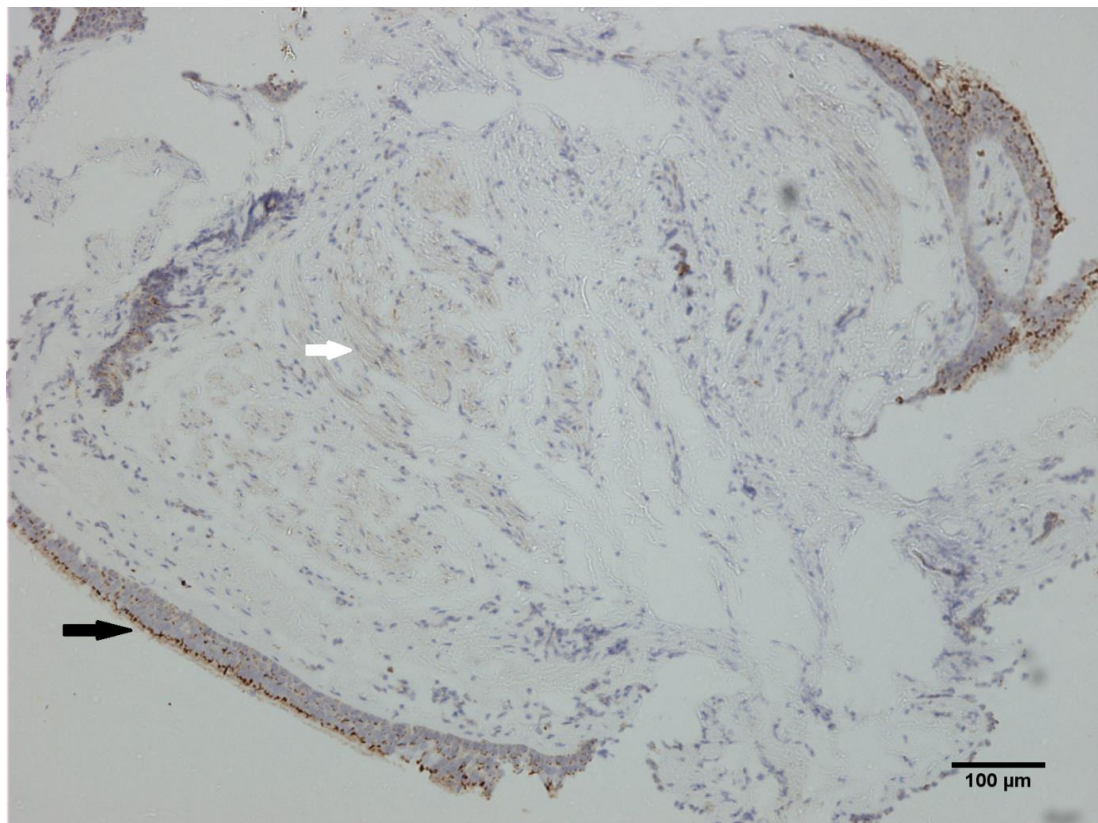


Figure 5A: Example of PAFR staining seen in non-smokers. Staining is seen on the airway epithelium, which showed normal pathology, as indicated by the black arrow. The white arrow indicates smooth muscle tissue in which staining was minimal in this sample. The other non-epithelial positive staining seen in the image is a bronchial gland.

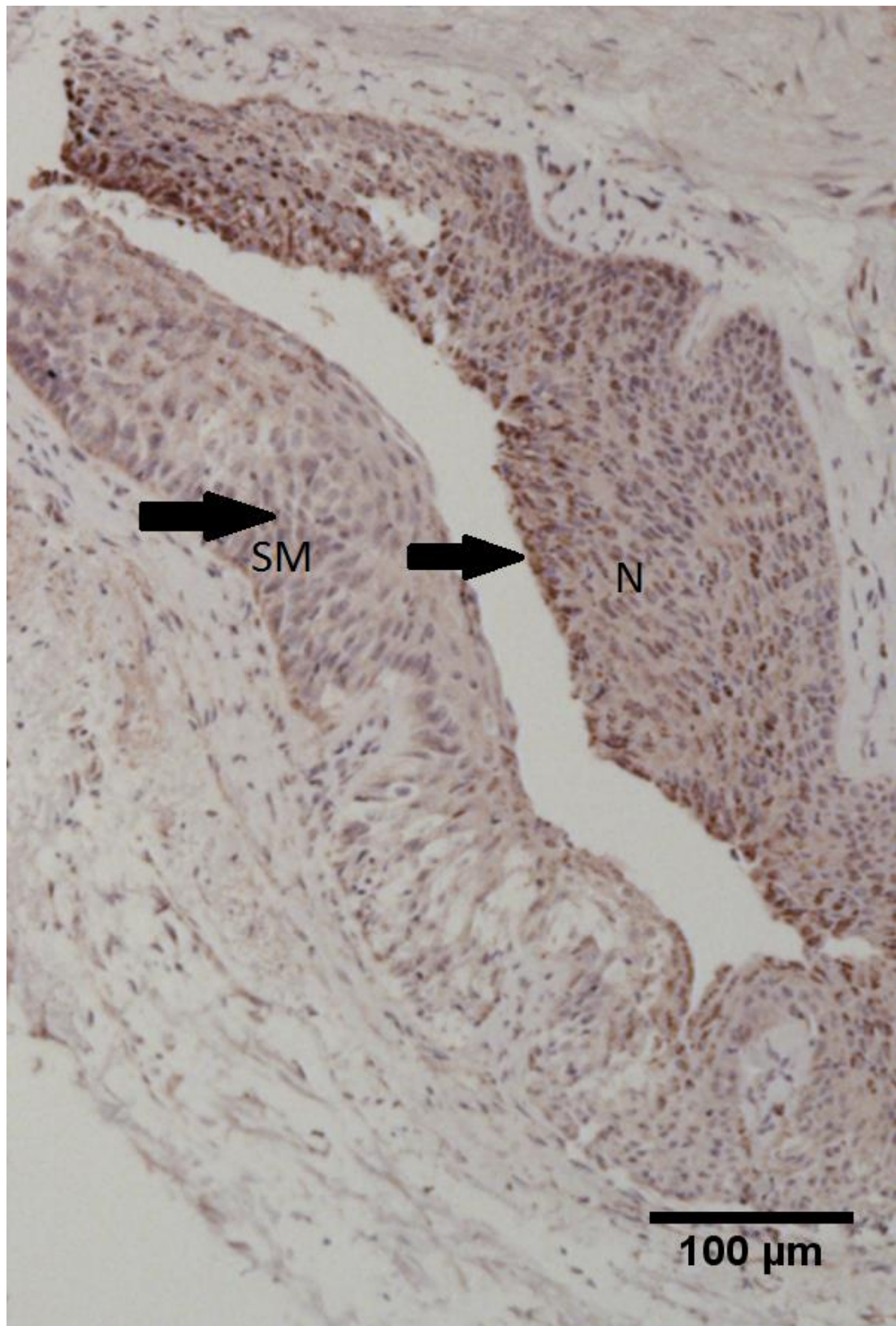


Figure 5B: Example of PAFR staining in smokers. PAFR staining was higher on the normal epithelium (N) compared to the squamous metaplastic epithelium (SM).

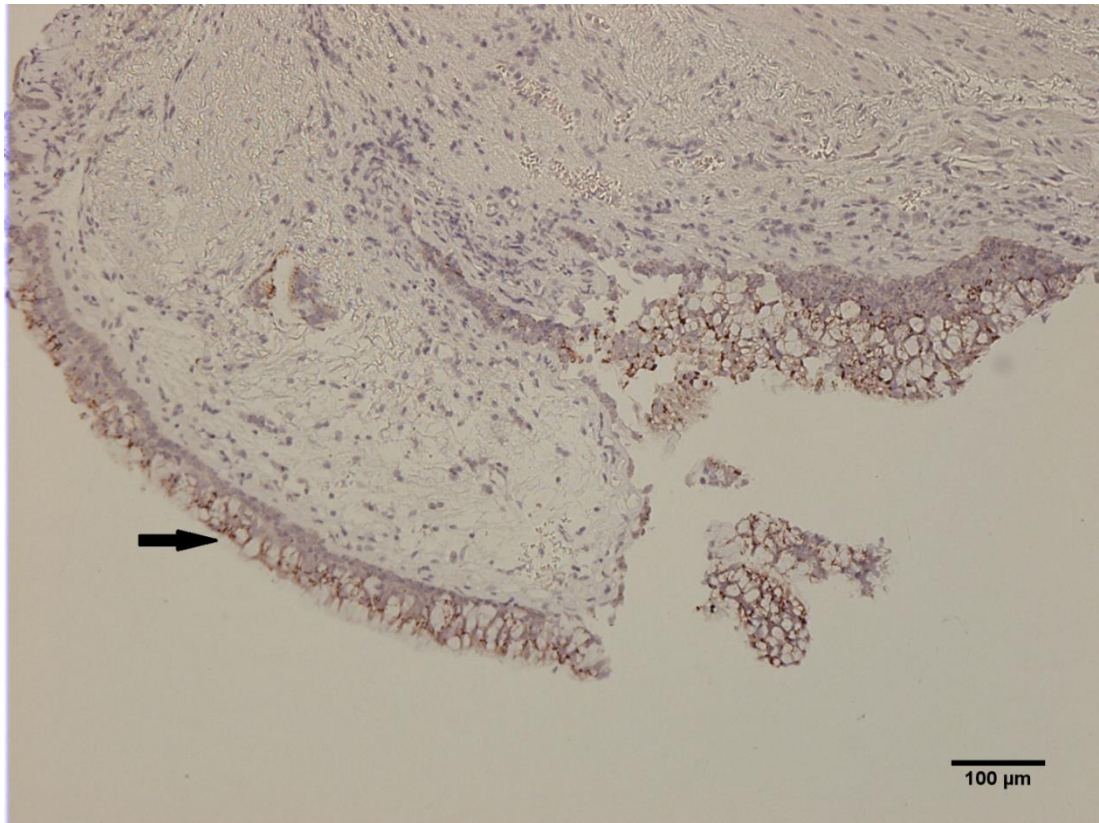


Figure 5C: Example of PAFR staining in smokers with COPD. PAFR staining was predominantly in the epithelium as indicated by the black arrow. Goblet cell rich epithelium as seen in this example was observed in both smokers and smokers with COPD.

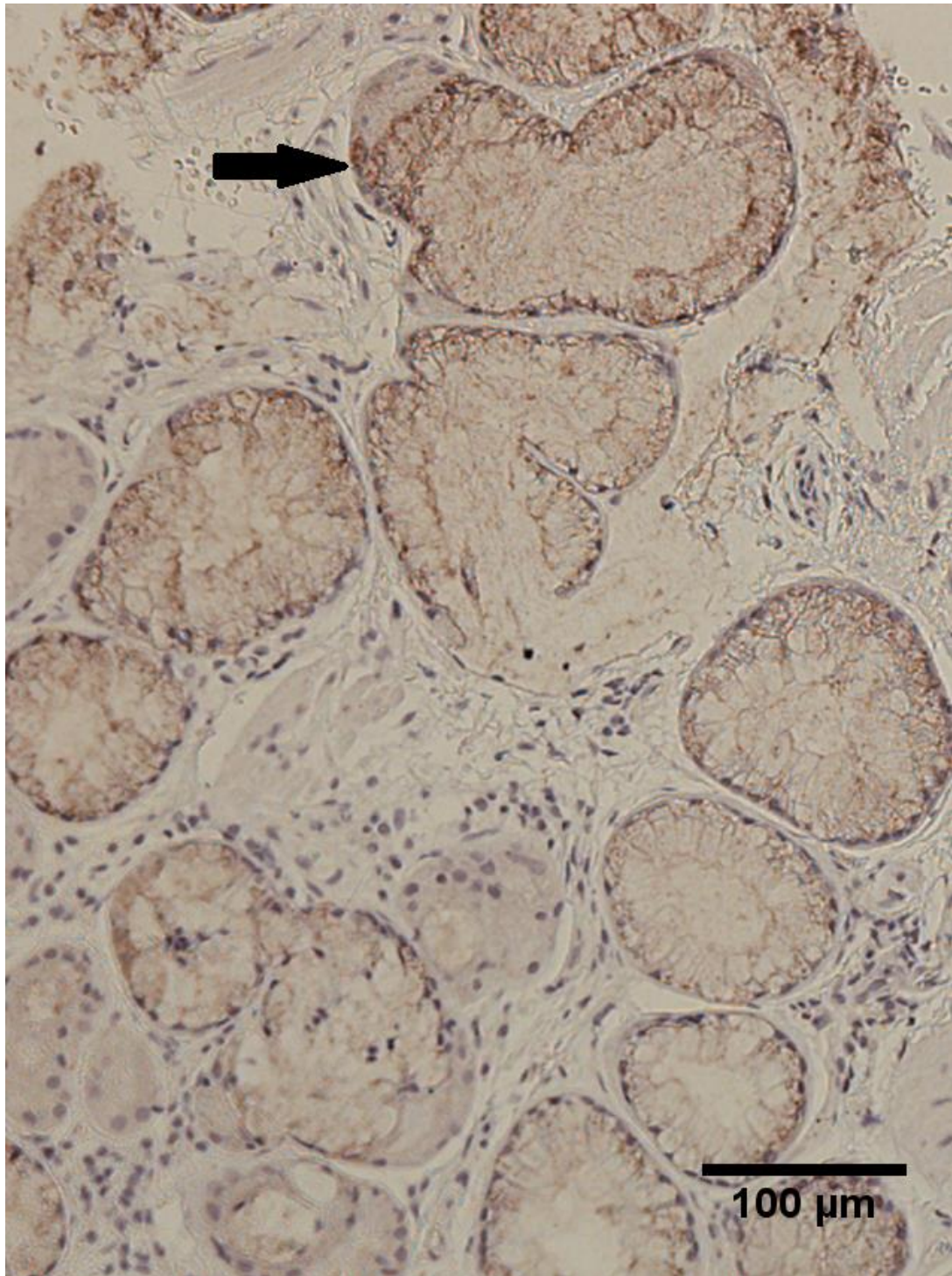


Figure 5D: Example of PAFR staining in the bronchial glands. PAFR positivity in the bronchial submucosal glands, as indicated by the black arrow, was seen in all subjects in whom the glands were sampled by the biopsy.

6.3 Conclusions

Qualitative analysis of the non-smoking control and welder lung samples available showed that the welder sample had more PAFR positive bronchial epithelial cells. These cells are situated at the air-tissue interface and hence are exposed to airway bacteria. To date this is the first time that PAFR expression and localisation has been assessed in a welder's lung. Taken with the functional evidence for a role of PAFR in WF-mediated lower airway vulnerability to pneumococcal infection presented in chapter 3, this observation may have important clinical implications. However, a well powered study in welders needs to be conducted in order to confirm this observation.

Cigarette smoking causes chronic respiratory diseases such as COPD. COPD patients are highly susceptible to respiratory infections. Rhinoviruses have been shown to be associated with COPD exacerbations and secondary bacterial infections are a common occurrence⁶⁰. Exposure of lower airway epithelial cells to cigarette smoke extract has been shown to cause a PAFR-dependent increase in pneumococcal invasion and adhesion. Additionally, a higher proportion of bronchial epithelial cells have been shown to express PAFR in smokers compared to healthy controls¹²³. Hence, it was hypothesised that baseline PAFR expression would be higher in smokers and smokers with COPD, compared to non-smokers. Indeed in this study it was found that smokers and smokers with COPD have higher PAFR mRNA levels compared to non-smokers. However, there was no significant difference in baseline PAFR expression between smokers and smokers with COPD. This suggests that smoking per se increases PAFR-dependent susceptibility to pneumococcal infection and that there is no association between reduced lung function seen in COPD patients and PAFR mRNA expression.

Immunohistochemical analyses of baseline PAFR expression in paraffin embedded bronchial biopsy samples showed that PAFR is predominantly expressed in the

airway epithelium and bronchial submucosal glands. Both these areas of tissue are exposed to air. This suggests that the changes in PAFR mRNA expression observed can be mapped to areas that are exposed to airway bacteria. This, taken with the *in vitro* evidence that exposure to CSE increases susceptibility of bronchial epithelial cells to pneumococcal adherence, supports the hypothesis that PAFR has a role in increased susceptibility of smokers and smokers with COPD to pneumococcal infections. However, the role of PAFR in susceptibility to pneumococcal infections in COPD patients is yet to be elucidated.

Areas of squamous metaplasia and goblet cell rich epithelium were seen in smokers and smokers with COPD. Squamous metaplasia (SM) is a type of epithelial cell remodelling associated with chronic irritation and goblet cell hyperplasia is a common feature of COPD¹⁵⁹. These observations are consistent with literature that suggests a role of PAFR in driving goblet cell hyperplasia in a guinea pig model of chronic exposure to cigarette smoke¹¹⁶. It was also observed that PAFR staining was lower in squamous metaplastic areas of the epithelium. The low quality of the samples available for immunohistochemistry analysis and the differential staining patterns seen in the different types of epithelium made it difficult to quantify changes in PAFR expression. Hence, only qualitative observations were made about the 21 samples available. However, taken with the evidence that PAFR is an entry receptor for pneumococci, the pattern of staining suggests that airway remodelling towards an SM phenotype may confer protection against pneumococcal infections. Further work is required to explore the implications of this observation.

Data presented in chapter 5 showed that rhinoviral infection of lower airway epithelial cells increased pneumococcal adhesion and invasion in a PAFR-dependent manner. Additionally, it has previously been shown that rhinovirus infection upregulates PAFR expression in respiratory epithelial cells and pneumococcal adhesion to these cells^{30,160}. Contrary to these findings, in this study

PAFR mRNA expression levels did not change after rhinovirus infection in non-smokers, smokers or smokers with COPD. Since baseline PAFR mRNA expression was higher in smokers and smokers with COPD, compared with non-smokers, it is hypothesised that baseline PAFR expression is at maximum possible levels and cannot be further upregulated by additional stimulation. Due to the lack of post-rhinovirus infection samples for immunohistochemical analysis it was not possible to compare PAFR protein expression before and after rhinoviral infection. Hence, it was not possible to test this hypothesis in this study. Further, the samples may have provided a better insight into paired changes pre- and post-rhinoviral infection in non-smokers. The paired changes in PAFR mRNA expression were not significant but there was a trend towards higher expression after rhinoviral infection. Further work is required to better understand how rhinovirus infection affects PAFR expression, *in vivo*, in cells exposed to airway bacteria.

Post-rhinoviral infection PAFR mRNA levels were compared between non-smokers, smokers and smokers with COPD. No significant differences between the groups were observed. However, there were trends towards higher expression levels in smokers with COPD compared with non-smokers and smokers with normal lung function. Again, the study was limited by the lack of post-rhinoviral samples for immunohistochemical analysis. However, it was found that 6 out of the 7 smokers with COPD from whom samples were available developed secondary infections by the ChoP-expressing bacteria, *S. pneumoniae* and *H. influenzae*. ChoP is a component of the bacterial cell wall which interacts with PAFR to facilitate bacterial invasion of host cells. No rhinovirus infected non-smokers or smokers with normal lung function developed secondary infection by ChoP-expressing bacteria.

A major limitation of this study is statistical power. This is because the samples analysed in this study were mined from another larger study from which a proportion of samples were unavailable⁶⁰. This study provides first insight into the expression

of PAFR in smokers with COPD. PAFR expression is elevated in smokers with COPD in cells exposed to airway bacteria at the air-tissue interface, compared with non-smokers. Further, in spite of a possible increase in PAFR expression in non-smokers after rhinoviral infection PAFR expression may remain elevated in smokers with COPD compared with non-smokers. This has significant clinical implications when taken with the observation that approximately 85% of smokers with COPD in this study developed infections by ChoP-expressing bacteria secondary to rhinoviral infection. Thus, data presented in this chapter further strengthens the case for therapeutic blocking of PAFR in smokers with COPD.

Chapter 7: Discussion

7.1 Background summary

Pneumonia is a leading cause of death worldwide and the main bacterial causative agent of pneumonia is *S. pneumoniae*. *S. pneumoniae* is a Gram positive bacterium which is asymptotically carried in the nose by a large proportion of the population³. The pneumococcal surface contains ChoP which is also part of the human platelet activating factor (PAF). It has been shown that the pneumococcus mimics PAF and binds its receptor, PAFR, thus co-opting human PAFR for entry into host cells⁸.

Viral infections, such as rhinovirus infections, exacerbate pneumonia by increasing susceptibility to secondary bacterial infections. The human rhinovirus has been shown to co-opt host receptors such as ICAM-1 and low density lipoprotein (LDL) for entry into host cells^{32,33}.

Exposure to airborne particulate toxins, such as PM₁₀ and cigarette smoke, has been shown to increase vulnerability to pneumococcal infection^{86,123}. One focus of this study was occupational exposure to particulate matter in welders. Welders have been shown to be more susceptible to pneumonia in several epidemiological studies^{4,142,143}. Cigarette smoking is highly prevalent amongst this population of workers and may further increase vulnerability as well as vulnerability to invasive disease¹⁴⁴. Little is known about the molecular mechanisms involved. The current Health and Safety Executive guidance in the UK is that there is an association between exposure to welding fume and risk of developing pneumonia but that employers should prioritise welding fume exposure control measures over offering employees the pneumococcal vaccine¹⁶¹.

The second focus of this thesis was smokers with COPD. Chronic smoking or exposure to indoor air pollution, such as biomass or wood smoke, may lead to chronic respiratory conditions and reduced lung function^{61–63}. COPD sufferers are

highly susceptible to viral and bacterial infections which exacerbate their condition. Rhinoviruses and *S. pneumoniae* are two of the commonly isolated pathogens in COPD exacerbations but the mechanisms that increase susceptibility of these patients to infections are poorly understood.

7.2 Questions addressed

In the context outlined above this thesis aimed to address the following questions:

1. Does exposure to welding fumes cause a PAFR- and oxidative stress-dependent increase in susceptibility to pneumococcal pneumonia?
2. Does exposure to welding fumes cause an increase in susceptibility to rhinoviral infection and to secondary bacterial infections?
3. Do COPD patients, who are highly susceptible to both viral and bacterial infections, have increased PAFR expression in the lung epithelium?

7.2.1 Question 1: Exposure to WF and susceptibility to pneumococcal pneumonia: role of PAFR and oxidative stress

Epidemiological studies have shown that welders of working age are more susceptible to pneumococcal infections but that this effect is reversible. This suggests that welding *per se* may increase susceptibility to pneumococcal infection^{4,142,143}. Smoking is a major confounder in these studies which also suggest that smoking may increase susceptibility of welders to invasive pneumococcal disease¹⁴⁴. To date no biological plausibility of increased vulnerability of welders to pneumococcal infection has been established and little is known about the molecular mechanisms.

PAFR is a known entry receptor for the pneumococcus in host cells. It has been shown that a mucolytic drug, S-CMC, which attenuates pneumococcal adherence to lower airway epithelial cells, also attenuates PAFR protein and mRNA expression in

these cells¹⁵. Additionally, work on the effect of other airborne toxins such as PM₁₀ and CSE on vulnerability of lower airway epithelial cells to pneumococcal infection has shown that a PAFR blocker attenuates this vulnerability. PAFR expression has also been shown to be higher in the lungs of mice exposed to cigarette smoke and in the lungs of smokers¹²³. This evidence suggests that PAFR may be a common receptor involved in particulate matter-stimulated vulnerability to pneumococcal infection in airway epithelial cells. Indeed the data in this thesis supports that idea and is the first evidence of a role of PAFR in WF-stimulated vulnerability of airway cells to pneumococcal infection.

Given that *S. pneumoniae* is commonly carried in the nasal cavity, it is of significance that exposure to WF increases susceptibility of nasal epithelial cells to pneumococcal infection. This finding raises important questions such as can pneumococci translocate across the nasal epithelial barrier into the blood stream? Does infection of the nasal epithelium lead to inhalation of the bacteria into the lower airway?

Like PM₁₀ and CSE, both types of WF used in this study i.e mild steel and stainless steel WF, are complex chemical mixtures. The two elements found in all three types of WF used in this thesis were iron and manganese. Chromium and potassium were contained in at least two of the three types of WF. Hence, the next steps for this work would be to dissect out the effects of the various metal components in this system in order to better understand differential effects of the different types of WF used.

A specific PAFR blocker was used in the *in vitro* functional studies. Additionally, PAFR protein expression was higher in lower airway epithelial cells after WF exposure. PAFR mRNA expression was also upregulated in the lungs of mice exposed to WF by inhalation and by intranasal inhalation. Further, higher PAFR

protein expression was seen in the lungs of a welder compared to a non-welder. While this evidence strongly suggests a role for PAFR in WF-stimulated vulnerability to pneumococcal infection *in vitro* and *in vivo*, the study has some limitations. First, although CV-3988 is a specific PAFR antagonist it is a synthetic agent and may have other, yet unknown, effects on cells. PAFR mRNA studies in cell lines proved inconclusive. It may have been useful to study the kinetics of PAFR expression in the cell lines to understand why this was the case. However, due to the availability of an animal model and some human tissue to confirm the PAFR expression findings PAFR mRNA studies in the cell lines were abandoned. It would be of interest to work with or develop a PAFR knockout mouse but time and financial consideration did not allow for this for this thesis¹⁶². Finally, the human lung samples available for PAFR protein analysis were mainly from smokers and ex-smokers. This confounded the analysis and so it was only possible to assess the effect of welding *per se* on PAFR expression in the lungs of one welder and one non-welder.

Similar to PAFR expression, oxidative stress has also been shown to be increased by exposure to inhalable toxins. Bronchial epithelial cells exposed to PM have increased ROS, IL-8 and IL-1 β release. These increased levels have been shown to be attenuated by NAC which is an anti-oxidant^{68,69}. NAC has also been shown to attenuate lower airway cell injury and pneumococcal adhesion to these cells caused by exposure to CSE¹²³. The literature reports increased free radical, ROS and NO production after exposure to WF^{139,141,155}. The data in this thesis, which show a role for the anti-oxidant, NAC, in attenuating WF-stimulated pneumococcal adhesion, provide functional relevance for oxidative stress in WF associated pneumococcal infections. Although ROS release was not measured in this system the data highlight the possibility of the involvement of other related mechanisms. It would be of interest to investigate the relationship between oxidative stress and PAFR expression in lower airway epithelial cells in the context of pneumococcal infections.

A mouse model of pneumococcal infection was used to assess whether exposure to WF *per se* increased susceptibility to pneumococcal infection. A major limitation of this model was the method of WF delivery. Ethical considerations only made it possible to deliver WF using intranasal instillation, which is not physiological. Lung samples from a study in mice exposed to WF by inhalation were available for PAFR mRNA analysis, as discussed above, but not for investigation of vulnerability to pneumococcal infection. The intranasal method of WF delivery made it difficult to calculate a physiologically relevant dose because to date there have been no studies which measure particle deposition in the alveolar and bronchial regions in welders. It is currently estimated that welders are exposed to $80\text{mg}/\text{m}^3 \cdot \text{years}$ of WF^{132,133}. Hence, the dose chosen was one that was tolerated by the mice by intranasal instillation under anaesthesia with minimum discomfort and that did not cause the BALF to turn brown 24 hours after instillation. A dose of $600\mu\text{g}$ was the highest possible tolerated by a single mouse by intranasal inoculation under anaesthesia. The only other mouse study in which the WF was delivered in a similar way used a total dose of $400\mu\text{g}/\text{mouse}$ delivered in $100\mu\text{g}$ instalments over 4 different days¹³⁴. In our low dose chronic exposure experiment we delivered a total of $600\mu\text{g}/\text{mouse}$ in $100\mu\text{g}$ instalments over 6 different days. Other studies in rats have used a total dose of $2\text{mg}/\text{rat}$ ^{139,145,155}.

The mouse studies showed that exposure to WF caused increased pneumococcal infection but did not cause invasive pneumococcal disease. Given that invasive disease was only seen in some smoking welders in epidemiological studies it may be of interest to show biological plausibility in mice exposed to both cigarette smoke and WF¹⁴⁴. This may help to further dissect out the differences in effects caused by WF *per se* vs. smoking + exposure to WF.

7.2.2 Question 2: Exposure to WF and vulnerability to viral infections and secondary pneumococcal infections

There is a body of literature which supports the idea that viral infections predispose to secondary bacterial infections^{30,43–45,51}. Of particular interest for this thesis, in the context of pneumonia, were the interactions between rhinovirus, which is frequently associated with exacerbation of COPD, and the pneumococcus. Since welders are more susceptible to pneumonia compared to the general population it was of interest to assess whether exposure to WF increases vulnerability to rhinoviral infection and subsequently to secondary pneumococcal infection.

The human rhinovirus co-opts host receptors such as ICAM-1 and LDL for entry into host cells³³. For this thesis human rhinovirus subtype A, serotype 16 was used. The entry receptor for RV-16 is known to be ICAM-1. ICAM-1 is known to be upregulated by viral infection in airway epithelial cells³³. Exposure of lower airway epithelial cells to mild steel WF, upregulated ICAM-1 expression in both cell lines used. This suggests that exposure to mild steel WF increases vulnerability of these cells to rhinoviral infection. This was confirmed by assessing the amount of viral mRNA in these cells after exposure to mild steel WF. However, with stainless steel WF, although increased viral infectivity was seen, the exposure to stainless steel WF did not upregulate ICAM-1 expression. Although the functional outputs from using the two types of WF were similar the mechanisms involved are not. This suggests that ICAM-1 is not the only receptor used by RV-16 to infect host cells and comes back to the question of understanding how the components of each type of WF contribute to the differential effects seen. This work was not expanded as its main aim was to show that exposure to WF increases susceptibility to rhinoviral infection in lower airway epithelial cells. However, current ideas for expansion include the use of an anti-ICAM-1 agent to test the role of ICAM-1 in WF-stimulated susceptibility to viral infection. An anti-ICAM-1 antibody was used to this effect but the large amount of

antibody required for the experiments coupled with the inconclusive data caused this avenue of enquiry to be put on hold. It is also of interest to study the effect of WF exposure on LDL expression in lower airway epithelial cells.

Rhinoviral infection of WF exposed lower airway epithelial cells increased their vulnerability to secondary pneumococcal infections. These data are of some significance to strategies to protect welders against pneumonia but have some limitations. As mentioned earlier in this chapter *S. pneumoniae* is carried in the nose in a large proportion of the general population³. Similarly rhinoviruses are primarily thought to infect the upper airway¹⁶³. Hence it is of interest to investigate the possibility that the pathogens are inhaled into the lower airway from infected nasal cells. The development of a cell culture system to model this is currently in progress. Another limitation of the study was in the modelling of the exposure to a low dose of WF for a longer period of time. With the cell culture system used in this thesis it was only possible to model the overnight exposure to a single dose (10µg/ml). This was because it was the highest dose tolerated by the cells. It may be of more physiological relevance to use a mouse model to study the effect of chronic exposure to WF on rhinoviral/pneumococcal infections.

Given that PAFR has a role in WF-stimulated pneumococcal infection of airway epithelial cells it was of interest to assess the role of PAFR in cells co-infected with rhinovirus and pneumococci. There is a body of evidence that supports a role for PAFR in viral/pneumococcal interactions. Pharyngeal cells exposed to poly I:C were more susceptible to pneumococcal infections, which was attenuated by a PAFR antagonist⁴³. Tracheal epithelial cells infected with rhinovirus were more susceptible to pneumococcal infections which were attenuated by a PAFR antagonist³⁰. Additionally, in mice infected with influenza a PAFR antagonist reduced lethality, lung injury and neutrophil recruitment^{48,49}. Finally, returning to the idea of a common role of PAFR in particulate matter-stimulated effects is a study in mice exposed to

diesel exhaust particles that had upregulated PAFR mRNA levels in the lungs¹⁴⁷. Hence, it is not surprising that CV-3988 attenuated WF- and rhinovirus-stimulated pneumococcal infection of lower airway epithelial cells *in vitro*.

Currently the field is open for studies to identify novel anti-rhinoviral agents due to the lack of reliable preventions or cures. Iota-carrageenan has shown promise in randomised control trials in adults and children in which a reduction in nasal viral loads has been seen. Additionally, the compound has proved to be safe in children down to 6 months of age and is approved for over the counter use³⁹⁻⁴¹. However, little is known about the molecular mechanisms of its anti-rhinoviral activity. Due to the focus of this thesis on pneumonia it was of importance to assess the effect of iota carrageenan on secondary pneumococcal infections in rhinovirus infected lower airway epithelial cells. Showing an effect of iota carrageenan on secondary pneumococcal infections in rhinovirus infected cells was the first step in its study. Future work includes developing a better understanding of how IC confers protection against rhinoviral infection and assessing timing of intervention. It is also of interest to investigate any effects of IC on PAFR expression in secondary pneumococcal infections.

7.2.3 Question 3: PAFR expression in COPD

COPD is a chronic respiratory condition characterised by reduced lung function. COPD may be caused by chronic exposure to indoor pollution such as wood or biomass smoke or from chronic smoking⁶¹⁻⁶³. Inflammation of the bronchi and damage of the small airways are common features of the condition. Additionally, there is evidence that COPD sufferers have an impaired immune response to bacterial and viral infections^{115,164}.

Rhinovirus infections are a common cause of COPD exacerbations and secondary bacterial infections are frequently reported. In a human model of experimental

rhinovirus infection it was observed that a significantly higher proportion of COPD patients developed secondary bacterial infections compared to smokers without COPD and healthy controls. The majority of these infections were caused by ChoP expressing bacteria such as *S. pneumoniae* and *H. influenzae*¹⁶⁵. Since, as previously discussed, ChoP on the bacterial surface mimics PAF, binds PAFR and thus aids bacterial uptake into host cells, it was of interest to assess PAFR expression levels in the samples from this study. Additionally, based on the observation that COPD patients are more susceptible to bacterial infections it was hypothesised that they have higher baseline expression of PAFR compared to smokers and healthy controls. This hypothesis was also driven by the evidence that smoking *per se* causes increased PAFR expression in the bronchial epithelium¹²³. Hence it was of interest to assess whether COPD has an additive effect on PAFR expression. Further, in a guinea pig model of COPD increased goblet cell metaplasia was seen in the epithelium which was attenuated by a PAFR antagonist¹¹⁶.

Bronchial biopsies from healthy controls, smokers with normal lung function and smokers with COPD were examined at baseline and 7 days post-rhinoviral infection for PAFR mRNA expression. Smokers and smokers with COPD had higher baseline expression of PAFR mRNA compared to non-smokers but there was no significant difference between smokers and smokers with COPD. This suggests that the higher baseline PAFR expression in both smokers and smokers with COPD is driven by smoking rather than COPD *per se*. To date this is the first time that PAFR mRNA expression was analysed in smokers with COPD.

Paired analyses of PAFR expression at baseline and 7 days after rhinovirus infection showed no differences in any of the groups. However, there was a trend towards higher expression in non-smokers after rhinovirus infection. This study was the first to look at the effect of a rhinovirus infection on PAFR expression *in vivo*.

There is *in vitro* evidence that PAFR expression and *S. pneumoniae* adherence is increased in rhinovirus infected nasal and pharyngeal cells³⁰. This thesis presents the first evidence of a PAFR-dependent association between rhinovirus infection and *S. pneumoniae* adherence to bronchial and alveolar epithelial cells, and thus functional relevance for increased PAFR expression in the lungs after rhinovirus infection. However, the lack of statistical power in this study and the patchy nature of rhinovirus infection *in vivo* made it difficult to draw conclusions about whether rhinovirus infection increases PAFR expression in humans¹⁶⁶.

Comparing PAFR mRNA expression between groups 7 days after rhinovirus infection it was found that there were trends towards higher expression levels in COPD patients compared to healthy controls and smokers. A larger study with better statistical power may be able to resolve any differences between groups, if they exist, but it is difficult interpret these data.

A limitation of studying PAFR mRNA expression in bronchial biopsies is a lack of information about which cells are expressing the mRNA, which is of functional relevance. Hence, it was of interest to assess PAFR protein expression in the biopsies using immunohistochemistry. As the samples were mined from a previous study, only a limited number of baseline samples were available for analysis. This made quantitative analysis difficult and so only qualitative observations were made about the samples. Goblet cell metaplasia, as observed in the previously mentioned guinea pig model of COPD, was seen in both smokers and smokers with COPD. Squamous metaplasia (SM), which is also a feature of COPD, was seen in both smokers and smokers with COPD. It was observed that areas of SM had lower PAFR staining compared to other types of epithelia. Whether this was a technical artefact or is due to a biological feature of the type of airway remodelling warrants further investigation. If areas of SM express lower amounts of PAFR it may be hypothesised that the type of airway remodelling is a protective mechanism. This

observation added to the difficulty of quantifying PAFR expression in these biopsies. A larger study with additional time points for both PAFR mRNA and protein expression seems a reasonable way forward for this work.

Qualitative immunohistochemical analysis of the baseline bronchial biopsies of healthy controls, smokers and smokers with COPD showed that PAFR protein expression was restricted to the bronchial epithelium and the submucosal glands. Both these components of the small airways are in contact with inhaled air and thus with inhaled bacteria. The bronchial mucosal glands are responsible for mucus production in the airways. The patterns of PAFR expression seen suggest that the increased baseline PAFR mRNA expression measured may be mapped to areas that lie at the bronchial air-tissue interface. However, the functional relevance of this finding remains to be tested. Increased adherence of *S. pneumoniae* to bronchial epithelial cells exposed to cigarette smoke extract has been shown before but a similar effect in bronchial epithelial cells from COPD patients has not been shown to date¹²³.

7.3 Conclusions and a proposed mechanism for the role of PAFR in PM mediated vulnerability to pneumococcal pneumonia

7.3.1 Conclusions

In summary, evidence for the following has been presented in this thesis:

- A functional role of PAFR in WF-stimulated susceptibility to pneumococcal infection in lower airway epithelial cells and in nasal epithelial cells. Additionally, PAFR expression has been shown to be higher in mice exposed to WF, which are also more susceptible to pneumococcal infection, and in a welder.
- A functional role for PAFR in rhinovirus- and WF+RV-16-stimulated secondary pneumococcal infection in lower airway epithelial cells. Iota

carrageenan has been shown to have anti-rhinoviral properties, with respect to secondary pneumococcal infection, in these cells but its effect on PAFR expression is yet unknown.

- Higher baseline expression of PAFR in the bronchi of smokers with COPD, compared to non-smokers, and mapping of PAFR expression to cells at the bronchial air-tissue interface. The functional relevance of these findings is yet unknown.

7.3.2 PAFR as an anti-infective target

By putting this evidence in the context of the literature the author of this thesis would like to propose the following mechanism (figure 1) for a role of PAFR in airborne toxin-dependent vulnerability to pneumococcal infection. Airway epithelial cells are stimulated by inhaled toxins or inhaled toxins+rhinovirus. This leads to the upregulation of PAFR which is controlled by the transcription factor C/EBP δ ¹⁰. The ChoP moiety of bacteria, such as *S. pneumoniae*, can then bind to the PAFR. The PAFR/pneumococcus complex is internalised in a clathrin dependent manner⁹. The complex stimulates the pro-inflammatory mediator NF- κ B which in turn activates the inflammatory response. This includes the release of defensins and cytokines such as IL-8 and IL-1 β ^{18,69}. The PAFR/pneumococcus complex is also recognised by β arrestin 1 which aids either its translocation across the epithelial barrier which may lead to invasive disease or its targeting to the cell surface which may lead to infection and colonisation⁹. It is proposed that PAFR may be a good anti-infective target in vulnerable populations such as welders and patients with COPD, which are chronically exposed to inhaled toxins.

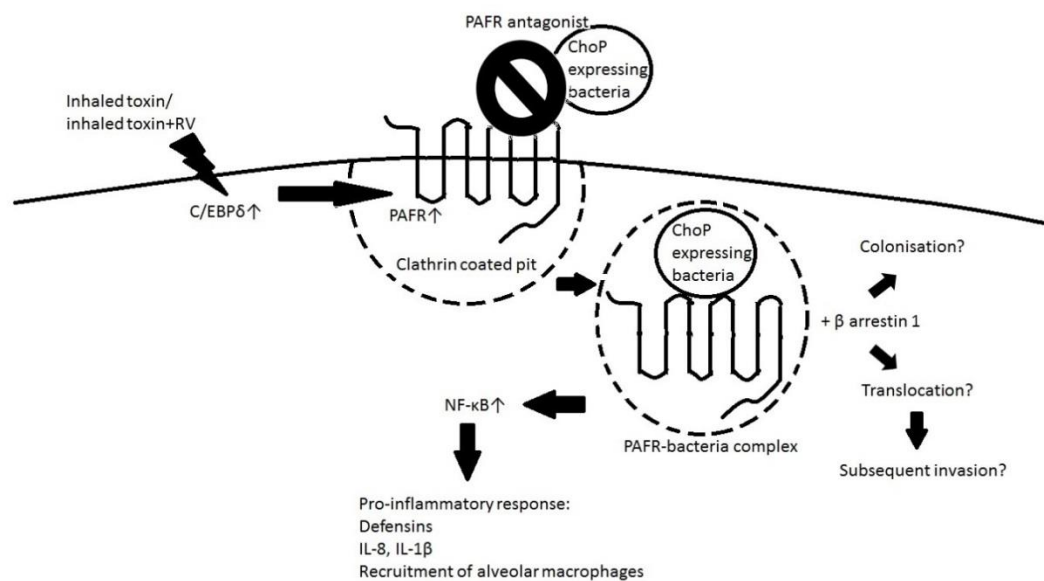


Figure 1: Proposed mechanism for inhaled toxin- or RV+inhaled toxin-stimulated vulnerability of lower airway cells to pneumococcal infection, which is PAFR dependent and hence may be blocked by a PAFR antagonist. The ↑ represents positive feedback. The ? represents as yet poorly understood elements of the mechanism which warrant further investigation.

Thus the evidence presented in this thesis, from both *in vitro* and *in vivo* studies, suggests that the use of a PAFR antagonist may be a viable prevention and protection strategy in vulnerable populations.

7.4 Future work

The data presented in this thesis are the first which show biological plausibility of an association between exposure to WF and vulnerability to pneumococcal infection. The work is also a step towards understanding the molecular mechanisms involved in conferring this susceptibility. To date offering welders the pneumococcal vaccine

is not a regulatory requirement¹⁶¹. Hence, some of the wider implications of the data include:

- A better understanding of exposure levels and better monitoring
- Making it a requirement for employers to offer the pneumococcal vaccine to high risk welders such as those who smoke or are older
- More research around PAFR as an anti-infective target and the development of PAFR antagonists

Although the proposed mechanism outlined above focuses on a PAFR antagonist it would be of interest to investigate whether molecules downstream of PAFR in its signalling pathway may be potential drug targets. It is also of interest to better understand the role of PAFR in both colonisation and invasion of the host and the different mechanisms involved. This is because invasion is only seen in a proportion of welders who smoke in epidemiological studies and no invasive disease was seen in the mouse model of pneumococcal infection used for this thesis. Additionally, colonisation and not invasion is relevant to chronic respiratory conditions such as COPD and cystic fibrosis.

Finally, although the focus of this thesis is PAFR, there are almost certainly other mechanisms and receptors involved^{19,24,25}. It was shown that oxidative stress mechanisms are also involved in increasing susceptibility of lower airway cells to pneumococcal infection after exposure to WF. However what these mechanisms may be was not explored in depth. A high throughput approach to identifying genes involved in the response of lower airway epithelial cells to WF followed by a pneumococcal stimulus may provide useful information about the PAFR and other pathways involved.

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